BIOANALYSIS OF SMALL MOLECULE PHARMACEUTICALS:

SIMULTANEOUS DETERMINATION IN BIOLOGICAL FLUID SAMPLES FROM MULTIPLE SPECIES BY THE MANAGEMENT OF MATRIX EFFECTS.

NICHOLAS PAUL GRAY

PhD

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NICHOLAS PAUL GRAY

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Research undertaken in the Department of Chemical and Forensic Science in collaboration with Sanofi-Aventis pharmaceuticals.

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Abstract

A strategy is described for method evaluation to manage the influence of endogenous compounds to induce bias in pharmaceutical quantification from blood samples of different animal species resulting from ionisation matrix effects. The approach introduces the possibility of simultaneous cross animal species calibration for ethical reasons using a simple indicative test, which also rapidly demonstrates the utility of a test assay in other matrices.

A quantitative Turboflow LC-MS/MS assay was evaluated using samples prepared in species matched controlled matrices with deuterated internal standardisation. The method was subsequently tested to assess the analysis of samples from multiple animal species using calibration samples from a single matrix origin. The object of this was to enable the substitution of analytical control samples in rodent plasma, reducing the plasma volume required, therefore the number of rodents used indirectly to support development studies. The method was unsuccessful due to concentration bias in identically prepared test samples. The bias origin was investigated using a fixed ratio solution of the analyte and its deuterated analogue with matrix test aliquots. The investigation identified non equivalent relative ionisation efficiency between compounds. The ratio method is proposed as an evaluation strategy for matrix effects causing non-equivalent response ratios.

The origin of this deviation was identified using a post column standard infusion test highlighting a region of ionisation suppression co-eluting with the analyte. Full scan acquisition analysis revealed co-elution of endogenous glycerophospholipids. The relative expression of these compounds between species was investigated, employing a precursor ion scanning method of a common product ion indicative of phosphotidyl choline compounds; the human diversity was also investigated. Distribution was found to differ between animal species, which was further tested by construction of a model using partial least squares regression which correctly identified the species origin of all non-primate species tested. The mechanism of differentiation between compound and deuterated analogue by endogenous phosphotidyl choline was proposed as micellar phase equilibrium following elution from the HPLC column.

A new Turboflow LC-MS/MS assay was optimised with attention to the resolution of glycerophospholipid interferences and retested for applicability between species. It was simultaneously applied to the analysis of small volume whole blood samples via dried blood spots on filter paper. Precision and bias were acceptable for the analysis of rat and mouse samples using human control plasma. Analysis of incurred *ex-vivo* samples from rats was successful in demonstrating equivalence between calibration sources.

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Finally my thanks go my parents and to my wife Helen and daughter Jasmine for supporting and motivating me throughout.

Authors Declaration

Work described in this thesis has been conducted exclusively by me, within the laboratories of Sanofi-Aventis, Alnwick Research Centre; I declare that this has not been submitted for any other award.

Parts of the research were conducted in support of pharmaceutical development studies on behalf of Sanofi-Aventis.

_____ _____

Nicholas Paul Gray Date

Statement of Ethics

No *in-vivo* studies were conducted exclusively for the conduct of this project. A core objective of the investigation was to evaluate bioanalytical strategies to support the reduction of the number of animals used indirectly in the generation of control matrices for use in support of bioanalytical determination of experimental pharmaceuticals in non-clinical species. This objective is in pursuit of the Sanofi-Aventis charter on the humane care and use of laboratory animals which states 'animals are used only when valid non-animal alternatives do not exist'. This project aimed to contribute to the development of valid non-animal alternative to the removal of blood from experimental animals to produce control matrix.

Any data from the analysis of samples drawn from studies involving non-clinical species have been conducted in conjunction with planned drug development study sample analysis, or following the completion of study sample analysis on samples scheduled for disposal. All bulk prepared analytical samples in animal plasma were prepared to support scheduled drug development study sample analysis.

Human blood products used for the purposes of this project were obtained from anonymised donors from a third party organisation and had been screened against antibodies for HIV, Hepatitis B and C. All experimental work was performed within the department of Global Metabolism and Pharmacokinetics, Sanofi-Aventis, Alnwick Research Centre, Alnwick UK in compliance with applicable health and safety practices.

Glossary of Terms

Descriptive Statistics

Mean Percent Difference (M%D) is used to describe the mean bias of a group of values as their deviation from a nominal or expected value. It is calculated by subtracting the mean experimental value from the expected value and expressing the difference as a percentage of the expected value.

$$\mathrm{M\%D} = \frac{Mean\ Experimental\ Concentration - Expected\ Concentration}{Expected\ Concentration} \times 100$$

Percent Coefficient of Variation (%CV) is used to describe the imprecision in a group of values, calculated by expressing the standard deviation as a percentage of the mean.

$$\mathrm{\%CV} = \frac{Standard\ Deviation \times 100}{Mean}$$

Inter-run Coefficient of Variation is used to describe run to run variability following multiple determinations of the same sample on multiple occasions. It is calculated as the %CV of mean calculated concentration results from several runs containing multiple determinations of samples of the same nominal concentration.

Intra-run Coefficient of Variation is used to describe the variability which exists within individual runs following multiple determinations of the same sample on multiple occasions. It is calculated as the mean of %CV values calculated from several runs containing multiple determinations of samples of the same nominal concentration.

Total Coefficient of Variation is used to describe the variability in a large dataset independently of individual runs and is calculated as the %CV of every individual concentration value of the same nominal concentration.

Physicochemical Parameters

Log P (or log $P_{oct/wat}$) is the logarithmic expression of the octanol-water partition coefficient (P) and is used as a measure of the hydrophobicity (or lipophilicity) of a compound by describing the partitioning of the compound at equilibrium between water and an

immiscible non-polar solvent. The partition coefficient is calculated for the neutral molecule therefore to determine the coefficient experimentally requires adjustment of pH ensure the compound was predominantly unionised.

$$\log P = log\left(\frac{[solute]octanol}{[solute]\ water}\right)$$

Log D (or log $D_{oct/wat}$) is the logarithmic expression of what is often described as the distribution coefficient (D). It is equivalent to log P as a function of pH and describes the hydrophobicity of a compound under test pH conditions.

OECD is the Organisation for Economic Cooperation and Development, an intergovernmental organisation in which representatives of industrialised countries in North America, Europe and the Pacific, as well as the European Commission, meet to co-ordinate and harmonize policies, discuss issues of mutual concern, and work together to respond to international problems. The work of the OECD related to chemical safety is carried out in the Environmental Health and Safety Division. A key document published by the OECD governing the conduct of pharmaceutical laboratory and analysis is 'Testing and Assessment; Principles on Good Laboratory Practice and Compliance Monitoring'.

Selected Reaction Monitoring (SRM) is the method of generating a selective ion signal on a triple quadrupole mass spectrometer by selecting a precursor ion using the first quadrupole, which is transmitted to the second quadrupole where fragmentation is induced. The product ion is transmitted to the third quadrupole where the major product ion is selected for detection.

1 Pharmaceutical Development

1.1 Overview of Pharmaceutical Development

Pharmaceutical development is a lengthy, extensive and expensive process. Of the hundreds of thousands of new chemical entities entering discovery research, typically one will reach the pharmacist. This follows a development programme lasting up to 15 years, involving hundreds of multidisciplinary teams of scientific and medical professionals and following a research and development budget approaching 1 billion US dollars. [1, 2]

The first stage in the development lifecycle is 'drug discovery research'. The process is devoted to identifying new compounds which have the potential to induce a pharmacological effect which may be applied to the improvement or prevention of disease. Methodologies in the research stage are high throughput; where hundreds of analyses are performed each day using techniques to facilitate automated sample preparation and rapid analysis. [3] Therapeutic targets are identified by bioinformatic technologies which use powerful computational resources to characterise endogenous chemical changes during the progression of a disease state by comparing diseased subjects with the healthy population. [4] Chemical entities with the potential to effect a change in the disease state are synthesised and tested *in-vitro* before evaluation of safety and efficacy by screening studies in animal models. Chemical compounds with the potential to be developed as drug candidates are taken forward into 'preclinical development'.

Preclinical development progresses the drug as a raw material into a preliminary pharmaceutical preparation suitable to conduct clinical trials. The physicochemical properties of the compound are studied and characterised while it is formulated by pharmaceutical scientists into a dosage form to accurately deliver the compound into the body appropriate to the intended clinical use. Drug disposition studies are conducted to model the effect of the body on the concentration distribution or metabolic transformation of the drug, while pharmacodynamic studies relate these to the observed effects on the body. Toxicologists determine the safety of the drug in nonclinical *in-vivo* models prior to administration in clinical trials. Preclinical development is a very selective process further refining the compounds nominated from discovery research. If the drug candidate

compound demonstrates promising potential for efficacy in therapeutic use with an appropriate margin for safety, it will progress in to the clinical development programme.

Clinical development is divided into 3 distinct stages as represented in Figure 1-1. During Phase I, the compound is dosed to a small number of healthy volunteers, initially at doses significantly below therapeutic levels and considerably below those associated with any toxic effect. The trials correlate the activity of the drug in the body with that seen in animal models at clinically relevant doses so tolerability can be assessed before the dose is increased towards the target range. Following completion of phase I studies, the drug can be dosed to a small population of patients to refine and develop the dose formulation in phase II and then enter phase II trials on a much larger population. In phase III, population effects may be studied and the efficacy of the drug is compared with alternative marketed products for the condition in blinded clinical trials. Following successful completion of the clinical trials the company will file the drug for registration with international regulatory agencies and obtain approval to market the drug. At this point, the drug is available to clinicians to prescribe to patients as a marketed product and the company have exclusive rights to sell it, although the research groups continue to study the drug for new indications and effects on the larger population.

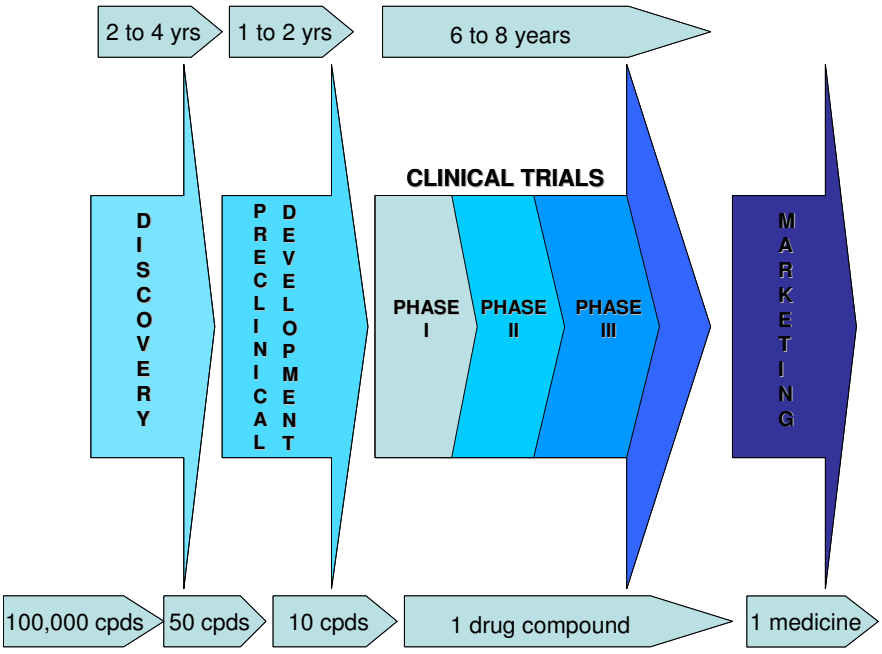


Figure 1-1; Stages of drug development

1.2 Disposition Science in Pharmaceutical Development

1.2.1 Pharmacokinetics

The study of pharmacokinetics models the concentration distribution of pharmaceutical compounds in the body to determine key parameters which are related to studies into safety and efficacy of the new compound. Pharmacokinetic studies are conducted throughout preclinical and clinical development. [5]

The pharmacokinetic profile is represented by Figure 1-2. In this simulation, following oral administration of the drug, concentration in plasma increases as the drug is absorbed into the blood compartment until it reaches a maximum concentration, when drug metabolism and elimination exceeds absorption to facilitate excretion via renal or hepatic clearance and removal from the body predominantly in urine or faeces. Drug may also be partitioned into body tissues. Pharmacological effects are proportional to circulating blood concentrations, often analysed and defined by plasma concentration therefore the therapeutic effect occurs at a given concentration range, above which toxicity may occur. Duration of therapeutic effect is consequently proportional to the period that the therapeutic concentration is maintained. The key pharmacokinetic parameters monitored in accordance with safety and efficacy studies are;

Bioavailability: This is the proportion of the administered dose which reaches the blood stream and plasma compartment to induce exposure to the body. It is affected by the physicochemical properties of the compound and characteristics of the product delivery route and formulation. [6]

Exposure ($AUC_{0\ to\ \infty}$): The area under the plasma concentration time profile from administration of the dose modelled to infinity. This is the characteristic measure of the exposure of the body to the dose administered and is proportional to the pharmacological effect. [7]

Maximum concentration (C_{max}): The maximum concentration achieved in the plasma compartment from a single dose. This is proportional to the intensity of the pharmacological effect. [8]

Time to maximum concentration (T_{max}): The time taken to achieve maximum concentration. This relates to the time delay between dose administration and the pharmacological effect. [8]

Half life ($T_{½}$): This measures the persistence of the drug in the body. It is proportionate to exposure. [6]

Clearance (Cl): This is the efficiency of the body in metabolism of the compound and elimination, principally by excretion into the urine or faeces. It is inversely proportionate to the half life. [6]

The study of pharmacodynamics relates the exposure to the pharmacological effect of the compound. The way that exposure parameters might relate to safety and efficacy are represented in Figure 1-3, where the therapeutic window defines the exposure at which the desired pharmacological outcome is achieved. If insufficient dose is administered, then no effect of the drug will be observed; however, if the dose level is too high and the therapeutic window is exceeded then toxicity may occur. [9]

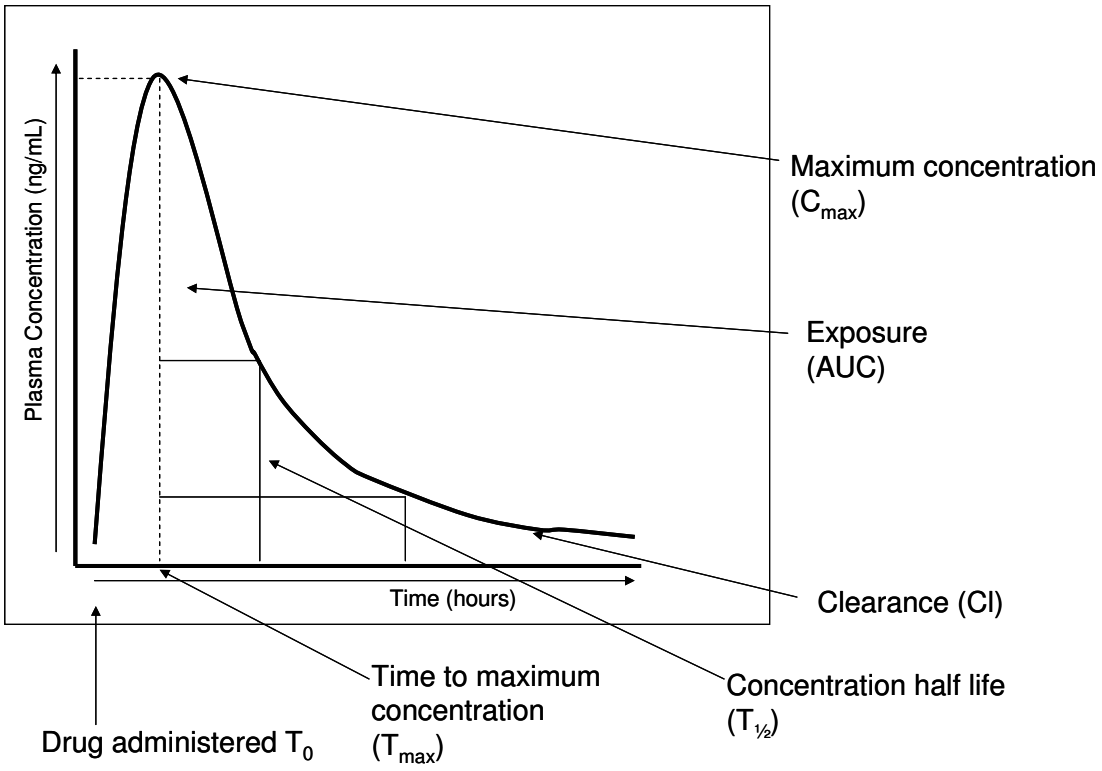


Figure 1-2; Oral pharmacokinetic profile and parameters

There are many factors which may influence the exposure of the body to the drug. [10] It is therefore necessary to not only model these pharmacokinetically but to control them via the ultimate pharmaceutical formulation and dose regime to ensure reproducible safety and efficacy of the product.

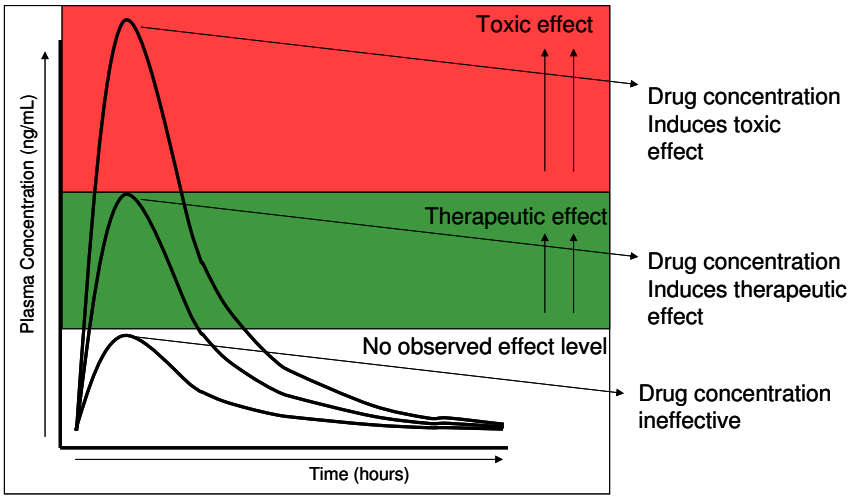


Figure 1-3; Relationship between exposure and therapeutic window

Other key pharmacokinetic factors which are evaluated are the effects of biological factors on the linearity of dose response and the impact of multiple dosing on accumulation and the induction of steady state when a constant concentration in the body is achieved in the therapeutic window without accumulating to the point where toxicity may occur [7, 11, 12] (Figure 1-4).

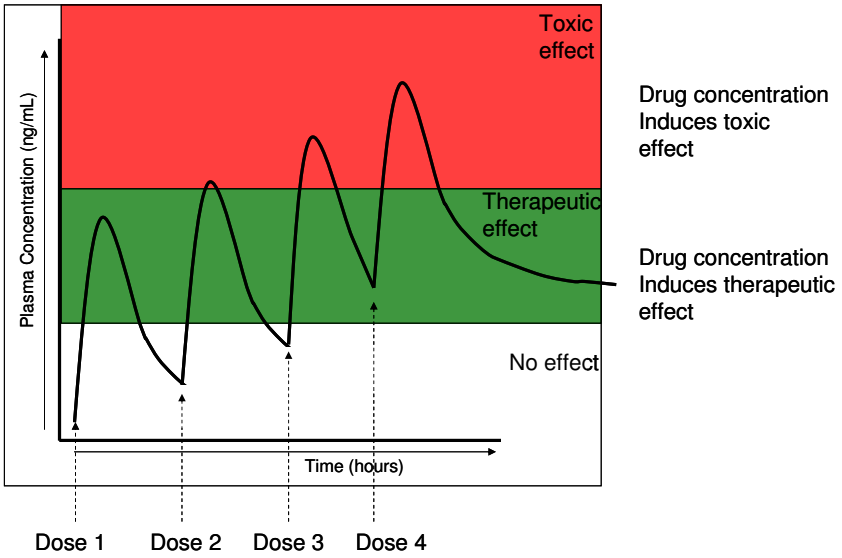


Figure 1-4; Effect of accumulation on exposure

1.3 Analysis of Pharmacokinetic Samples

1.3.1 Bioanalysis

Drug pharmacokinetic parameters are calculated from circulating concentrations of the compound in plasma or blood. Much of the drug compound circulating in the plasma component may be bound to circulating plasma proteins. [13, 14] It is the unbound drug in circulation which is pharmacologically active; however, the total drug in plasma is the concentration taken to represent pharmacokinetics and pharmacodynamics, so with the exception of specific studies concerned with protein binding, this is the concentration measured. The plasma constitutes 55% of blood volume and is derived experimentally from the supernatant following centrifugation of whole blood in the presence of anticoagulants in the region of 3000 rpm. The plasma contains 90% water, the remainder being composed of proteins, hormones and salts listed in Table 1-1 as well as co-administered xenobiotic compounds. [8]

In the pharmaceutical industry, pharmacokinetic bioanalysis is performed extensively by HPLC-MS/MS following extraction from biological fluid. [15] The first analytical problem in the development of bioanalytical methods is the removal or macromolecular interferences to prevent blockage of the analytical column. Historically, many methods followed crude protein precipitation sample preparation techniques, involving de-proteination of the samples using 2 to 4 volume equivalents of acetonitrile or methanol to cause protein denaturation. [16, 17] The enhanced signal specificity of triple quadrupole mass spectrometry reduced the necessity to resolve interferences to prevent direct chromatographic interferences. Protein precipitation protocols were also extremely generic since they were largely independent of analyte physicochemistry. However, problems were encountered due to unmonitored components in the precipitated sample co-eluting with the analyte and giving rise to significant matrix effects. [18] Liquid-liquid extraction into an immiscible solvent is a more selective therefore cleaner extraction technique less prone to matrix effects, but less generic and more difficult to automate so practically producing lower throughput of samples. The first stage of a reliable bioanalytical method is therefore optimally a more selective extraction of the analyte group of interest from matrix components.

Component	Function
Serum albumin	The most abundant plasma protein, binds calcium
Serum Globulins	
α-globulin	Binds thyroxin and bilirubin
β-globulin	Binds iron, cholesterol, vitamin A, D and K
γ-globulin	Immunological antibody, also binds histamine
Prothrombin	Promotes blood clotting
Fibrinogen	Blood clotting protein
Enzymes	
Mineral ions; Na^{+}, K^{+}, Ca^{2+}, Mg^{2+},$H_nPO_4^{n-}$, SO_4^{2-}, HCO_3^{-}	

Table 1-1; Major regulated components of blood plasma

1.3.2 Solid Phase Extraction

A potentially selective extraction technique is solid phase extraction. [19, 20] This employs the loading of the plasma sample onto alkyl modified silica or polymer based stationary phase packed into a disposable polypropylene column to achieve complete adsorption of the analyte onto the sorbent material. The column is then washed to remove interferences with the analyte attached to the stationary phase *in-situ* to selectively remove small molecule chemical interferences while macromolecules are filtered at the top of the sorbent bed. The analyte is later eluted by passing an appropriate solvent through the column and the eluent further processed for transfer to the HPLC analytical system. This technique is extensively commercialised, with a range of sorbent chemistries available through alkyl ligands to ion exchange media and mixed mode chemistries [19, 21] both off-line (Figure 1-5) and in-line (1.3.3).

A summary of solid phase extraction chemistries and the mode of extraction used for the recovery of small molecule pharmaceuticals from plasma for analysis by LC-MS obtained from articles published in between 2009 and September 2010 (obtained referencing plasma, solid phase extraction and chromatography) is summarised in Table 1-2. Reversed phase polymeric sorbents were most widely used in both on-line and off-line modes, owing partly to the more generic application of such a widely retentive media. Reversed phase polymers were also widely used in mixed mode extraction chemistry.

	Chemistry	Mode	Material	Refs	Count
Polymeric	Reversed Phase Polymer	ON-LINE	Oasis HLB	22, 23	5
			Supelco HiSep	24	
			Hysphere resin GP	25	
			Turboflow Cyclone	26	
		OFF-LINE	Oasis HLB	27-45	21
			Varian Plexa	46	
			HyperSep	47	
Mixed Mode	Mixed Mode Reversed Phase Polymer- Anion Exchanger	ON-LINE	Oasis MAX	48	1
		OFF-LINE	Oasis MAX	49	2
			Oasis WAX	50	
	Mixed Mode Reversed Phase Polymer- Cation Exchanger	OFF-LINE	CleanScreen DAU	51	7
			Oasis MCX	52-56	
			Strata XCW	57	
Ion Exchange	Cation Exchanger	ON-LINE	Monolith	58	2
			Spherisorb SCX	59	
		OFF-LINE	Argonaut SCX	60, 61	4
			Bondelut PBA	62	
			Isolute PRS	63	
Alkyl bonded phase	C2	OFF-LINE	Isolute	64	1
	C1	ON-LINE	Kromasil	59	1
	C8	OFF-LINE	BondElut certify	65	2
			Isolute	66	
	C18	ON-LINE	Lichrospher	67	6
			Zorbax	68	
			Kromasil	69	
			Turboflow	70	
			Biotrap 500	71	
			Hysphere	72	
		OFF-LINE	Bond Elut	73	4
			AccuBond	74	
			Bakerbond	75	
			SepPak	76	
	$(CH_2)_3CN$	ON-LINE	Isolute	77	1
		OFF-LINE	Supelco	78	

Table 1-2; Literature review of SPE modes and chemistries for small molecule pharmaceutical recovery from plasma for LC-MS 2009 to August 2010

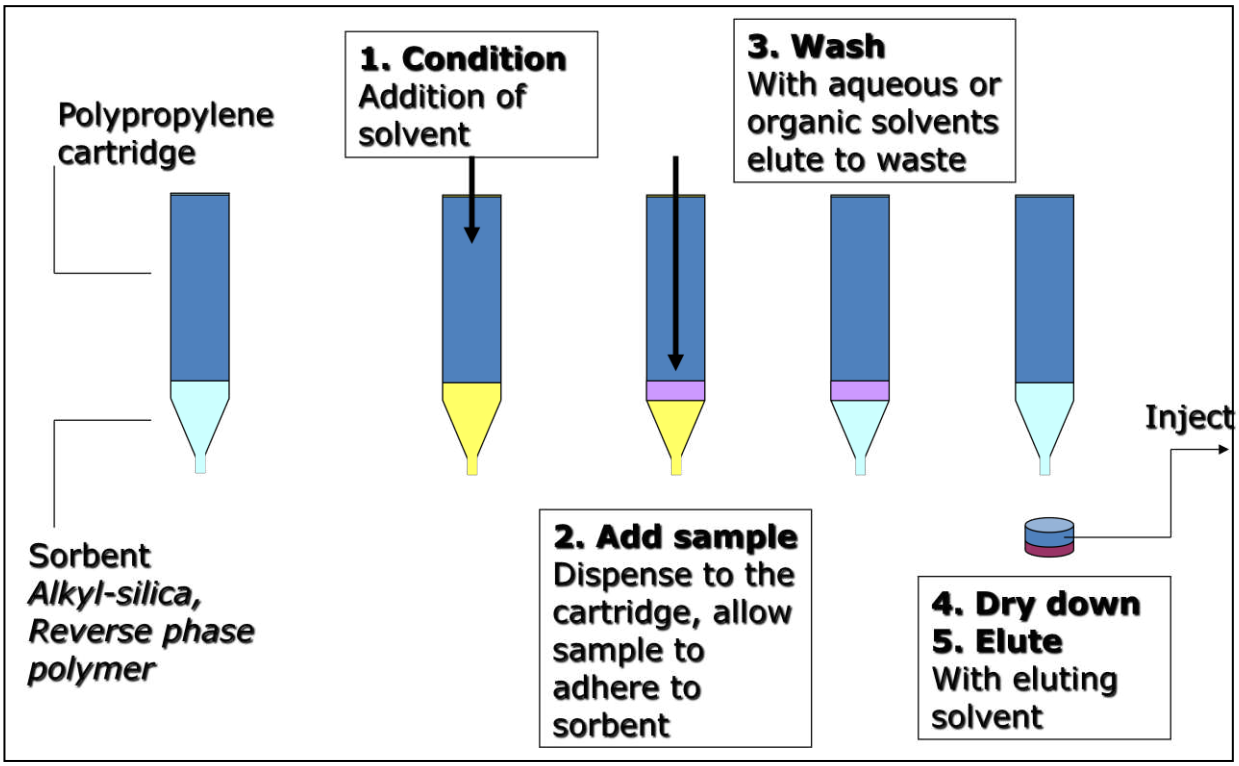


Figure 1-5; Schematic of off-line solid phase extraction

The large range of SPE chemistries commercially available enable a high degree of specificity, while microtitre plate formatting and robotic liquid handling platforms increased throughput and automation of SPE protocols. Interaction of the analyte with the stationary phase proceeds via polarity interactions on reversed phase alkyl modified silica or ion exchange on ionic surfaces, or in mixed mode sorbents (those containing both alkyl modified silica together with an ion pairing reagent). In addition to alkylated silica, polymeric sorbents with similar reversed phase properties are also used.

1.3.3 In-line Solid Phase Extraction

The solid phase extraction principal was modified to enable the repeated use of sorbent to extract multiple samples by formatting them into packed columns, with the aim of combining extraction and analysis into a single process. [79, 80] This is done by enabling extraction and retention of the analyte on the SPE pre-column and elution and transfer onto a second analytical HPLC column for separation and quantification. Modification of the sorbent particles sterically or geometrically prevents fouling by macromolecular species in the analysis mixture enabling reconditioning of the column between injections. These packed columns are generally referred to as restricted access media (RAM) columns. [81]

The first restricted access column was developed by Pinkerton et al. [82] in 1986 featuring standard chromatographic particles packed into a steel column, where access restriction to the sorbent was achieved either by replacing the external surface of the particle with an inert coating of the tripeptide Gly-Phe-Phe (internal surface reversed phase; GFF ISRP, Figure 1-6). Using the same principle a later modification was developed by Regnier and Dessiliets by bonding an inert polymeric 'mesh' of polyethylene glycol over the surface of the particle (semi permeable surface; SPS, Figure 1-7). [83, 84]

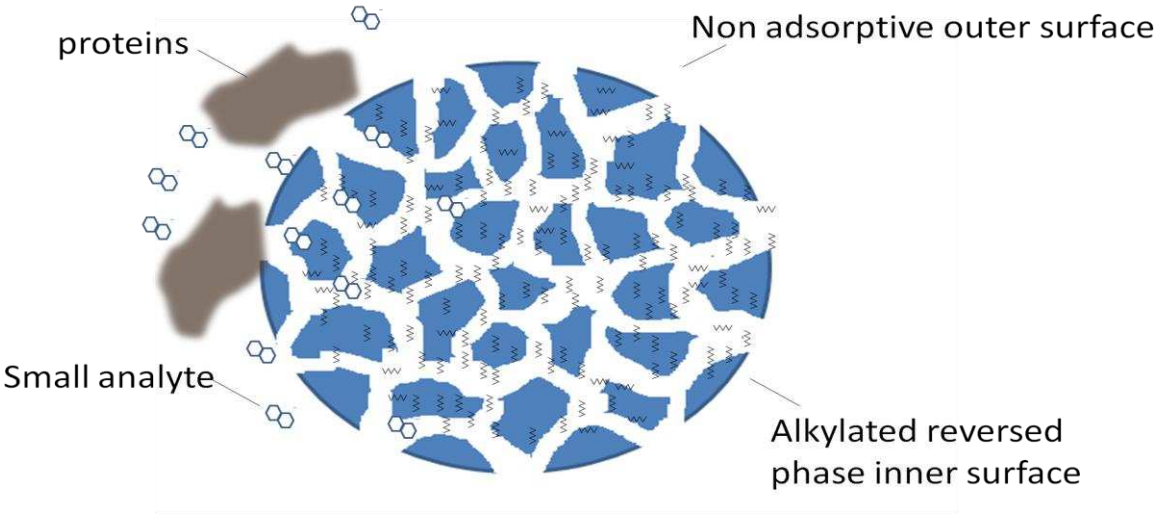


Figure 1-6; ISRP particle

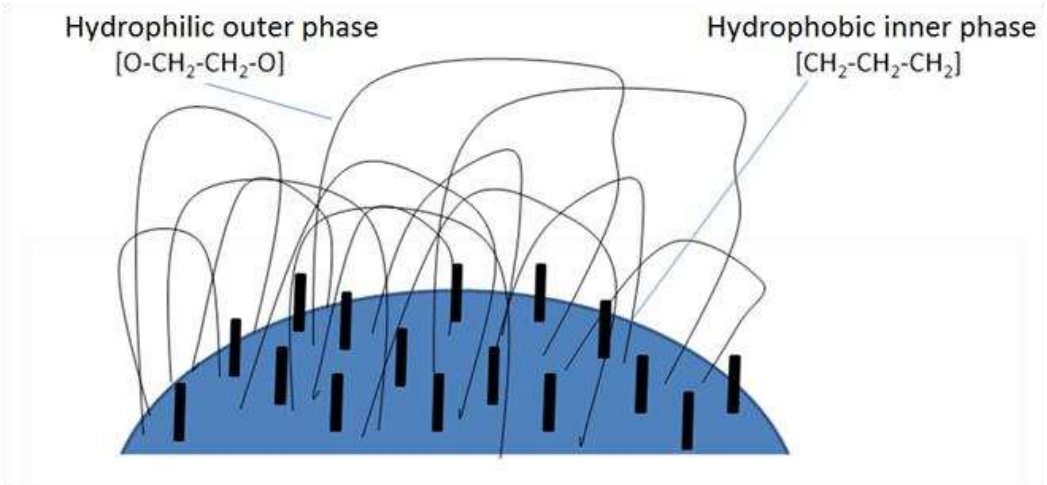


Figure 1-7; SPS particle

In both columns, the analyte is injected directly as a plasma sample towards the detector while the macromolecules flow over the surface and elute in the void volume of the column,

analytical gradients could then be applied to chromatograph the analyte as a standard HPLC injection. In practice, blockage of the column was found to be problematic, making analysis costly while the analyte retentive chemistry for single column injection was limited. [85] The columns were also used as pre-columns for multidimensional column switching methods enabling the injection and removal of macromolecules while the analyte was later eluted or further chromatographic separation done by liquid chromatography. [86]

The multidimensional approach was further optimised by the production of short, robust pre-columns dedicated to sample cleanup for column switching to analytical HPLC columns. An advantage of the coupled column approach for bioanalysis was that sample enrichment on the second column was enabled to support achievement of low limits of quantification, in addition to a higher loading capacity while column lifetime was extended. The simultaneous removal of macromolecules and analyte separation on the same column became a sequential process; however, since cleanup of the extraction column could be performed simultaneously with chromatographic separation on the second, there was no significant loss of throughput efficiency. The columns were prepared using the technique of enzymatic cleavage of the alkyl ligand from a bonded silica chromatographic particle and replacement by a biochemically inert barrier first demonstrated by Pinkerton. [82] Alkyl diol groups (Merck ADS) [85] or α_1 -acid glycoprotein (Biotrap 500) [87] were bonded to the cleaved silica of the external surface of the particle, leaving a reversed phase surface within the particle accessible only to analytes small enough to permeate the particle (15 kDa cut off).

An extensive range of chromatographic sorbent chemistries are available with the introduction of mixed mode particles. [88] Boos and Grimm categorised the available restricted access media columns into 4 types, [85] depending on exclusion barrier or surface topochemistry (Table 1-3). ISRP GRR (II) is an example of the physical exclusion where the reversed phase sorbent has been removed from the outer surface of the particle while BioTrap 500 is excluded chemically by steric restriction caused by α_1 -acid glycoprotein surface coverage. In each of these examples, the surface topochemistry is dual, the particle surface being functionally different to the inner pore surface.

TYPE	Exclusion Barrier	Surface Topochemistry	Commercial Products
A	PHYSICAL	UNIFORM	ChromSpher 5 Biomatrix (Chrompack)
B	PHYSICAL	DUAL	ISRP GFF (II) (Regis) LiChrospher ADS (Merck KGaA)
C	CHEMICAL	UNIFORM	Hisep (Supelco) Capcell Pak MF (Shiseido)
D	CHEMICAL	DUAL	Ultrabiosep (Shanon) BioTrap 500 (ChromTech) SPS (Regis) MAYI-ODS (Shimadzu)

Table 1-3; Boos classification of restricted access materials

A modification of RAM columns was the development of large particle support chromatography (LPS) columns [83, 89] where macromolecules are excluded by size using hydrodynamic properties of the mobile phase rather than steric restriction. The columns are constructed with a 30 - 50 µm particle size packed into 0.5 to 1.0 mm i.d. columns. The most prominent example of these columns is Turboflow (Thermo). [90, 91] LPS columns use a range of column chemistries in 50 µm particle sizes packed into steel columns. For a 1.0 mm i.d. column operating at a flow rate of 5 mL/min, the combination of flow rate and particle size enables the establishment of 'turbulent flow' characteristics, as defined by Quinn and Takarewski. [92] Eddy formation between stationary phase particles promotes radial diffusion across the column to reduce the effects of frictional drag against the column walls. This contributes to the band broadening observed as flow rate increases in the laminar flow region according to the Van Deemter relationship. Back pressure across the column is also reduced. The resulting flat fronted fluid profile is represented in Figure 1-8 in comparison with the less uniform flow profile observed in the laminar flow region; in consequence band broadening is relatively low and the sample is transferred to the front of the HPLC column in a tight band.

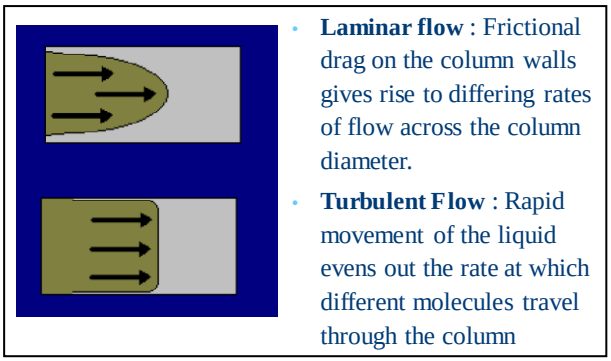


Figure 1-8; Flow profiles in laminar and turbulent flow

In the reduced equation of the Van Deemter relationship (Figure 1-9) within the laminar flow region, the C term is dependant on axial diffusion. The optimum (minimum) value of height equivalent of theoretical plates (H) in the laminar flow region is observed at relatively low flow rate, but increases with band broadening as flow velocity is increased since slow axial diffusion means that frictional drag to the tube walls (or particle surface) distorting the moving band. As flow rate increases, beyond the laminar zone, diffusion and mass transfer occurs more rapidly and the significance of the C term diminishes. Characteristically, the flow in the turbulent region takes on a more parabolic profile as frictional interactions with the vessel walls are less significant (Figure 1-8).

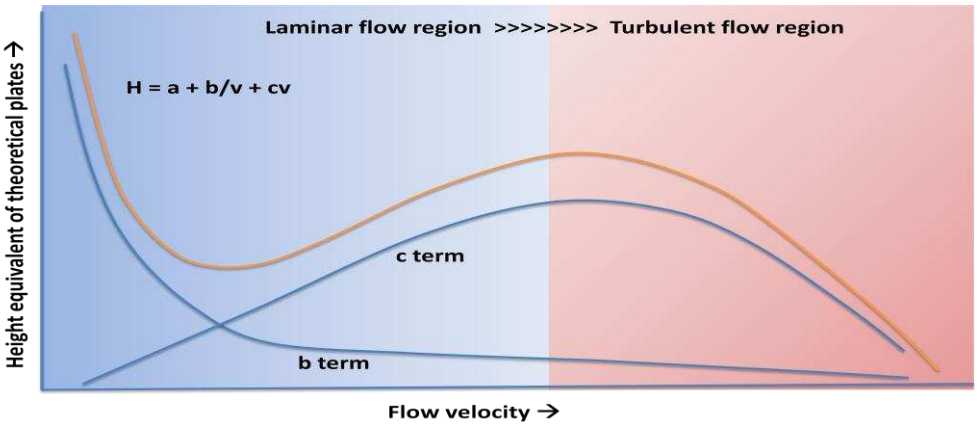


Figure 1-9; Illustrative representation of the Van Deemter relationship between linear velocity and height equivalent of theoretical plates

The advantage of this flow profile for extraction of small analytes from macromolecules is that while mass transfer of small molecules into the stationary phase is efficient, the eddies generated prevent macromolecules from accessing the pores so they are channelled to waste via the larger interstitial spaces. The profile restricts the access of macromolecules to the stationary phase since the spatial restriction close to the particle surface reduces the eddy formation so that mass transfer is limited by molecular diffusion, the rate of which is

proportional to molecular mass.[92] A practical advantage of extraction at high flow rate is that the separation occurs rapidly so speed of analysis is high which is favourable to sample throughput and relatively large volumes can be injected onto the column. As with other direct injection methods discussed, elimination of additional transfer steps aids sample conservation, especially where small sample sizes are available so that the maximum recovery of mass on the analytical column is attainable from the analytical aliquot taken.

1.3.4 Chromatographic Separation

The functionality of chromatographic separation is to provide a physical method of separation in which two components to be separated are distributed between two phases, one of which is stationary, while the other moves in a definite direction. This forms part of the definition accepted by IUPAC.[93] In the case of liquid bioanalytical separations, the chemical diversity and complexity of the sample mixture necessitates the separation of the analyte of interest from a very large number of defined and undefined co-eluents, for example drug metabolites, impurities, endogenous molecules, xenobiotic compounds, concomitant medications or contaminants resulting from sampling such as anticoagulants or plasticising agents.

As described in 2.1, the chromatographic separation in many bioanalytical methods typically involves two stages of liquid chromatographic separation. The first, during initial extraction where analytes that are large are excluded from chromatographic retention by some form of steric or dynamic restriction, or unretentive components are eluted to waste while the analyte and compounds with broadly similar structural and polar characteristics are retained. This has been described as 'digital' chromatography since upon contact with the chromatographic sorbent, analytes are either retained with total affinity or unretained and there is no migration through the column. The second chromatographic separation involves more specific separation across very pure sorbents under high pressure (typically 80 to 350 bar). Due to the aqueous nature of the samples injected, and primary extraction, this separation is reversed phase, either under isocratic conditions, or following application of an elution gradient, to ensure that the analyte migrates through the column at a rate which infers sufficient specificity from other compounds in the mixture injected (or switched from the primary extraction column).

Specificity and selectivity

Since quantitative analysis is achieved using mass spectrometers with a high degree of specificity, chromatographic selectivity has been considered less important than that required for less selective detection techniques such as HPLC-UV,[23] since there is far less incidence of chromatographic visible interference or baseline disturbance. The main function of the HPLC separation in this case is to concentrate the analyte into a narrow band during migration through the column which will elute as a sharp peak enabling efficient integration and maximising signal sensitivity. A rapid cycle time from sample injection to peak elution is important in the context of pharmacokinetic bioanalysis particularly in clinical studies as high sample throughput is necessary. However, co-elution of unseen components can give rise to less visible but equally undesirable effects of ionisation suppression (or enhancement) as described in section 2.1. It is therefore necessary that the HPLC separation achieves an adequate capacity factor (k'>5, so chromatographic retention infers sufficient separation from the solvent front and co-injected species) without losing the efficiency benefits of rapid separation.

Choice of mobile phase

There are a number of restrictions on selection of mobile phase chemistry due to the nature of the wider analytical system. The mobile phase in reversed phase HPLC consists of two components, the organic and aqueous solvents. In combination, the mobile phase should be compatible with the analyte, the analysis mixture, the stationary phase and the mass spectrometer as well as exhibiting more polar nature than the alkylated stationary phase.

The solvent must be relatively non-polar but water miscible, with a sufficiently high boiling point and suitable viscosity. Chlorinated solvents are unfavourable due to safety and environmental concerns and more highly flammable solvents are also avoided. Typically, methanol or acetonitrile are used as the solvent of choice. Buffer species is important not just in respect of buffering capacity but also in other chemical characteristics. Primarily, it must be volatile for compatibility with the mass spectrometer; therefore, salts of organic acids are favoured as formate or acetate.

The pH of the buffer influences not only the analyte but also the stationary phase. Since the stationary phase is alkyl modified silica, incomplete alkylation leaves free silanol groups on the surface of the stationary phase which, being acidic in nature dissociate under neutral or basic conditions, leaving an exposed anionic surface. There is therefore the potential for secondary interaction of analytes by cation exchange mechanisms with uncapped silica under basic conditions. This may give rise to loss of chromatographic peak shape by tailing. The use of buffers with pH values greater than pH 7 also tends to degrade the column by stripping the alkyl ligand.

A review of mobile phase components and their concentrations published in the Journal of Chromatography B volume 878 issues 1 to 26 (2010) is summarised in Figure 1-10 to Figure 1-12 and tabulated in Appendix 1. This review revealed that although pure water was occasionally used as aqueous mobile phase, the most frequent salts were ammonium salts of formic or acetic acid or acidification using the free acids only with the most frequent composition containing formic acid only (between 0.001 and to 2 % v/v). The only organic mobile phases reported during this period were methanol or acetonitrile or a mixed composition of the two with acetonitrile used most frequently.

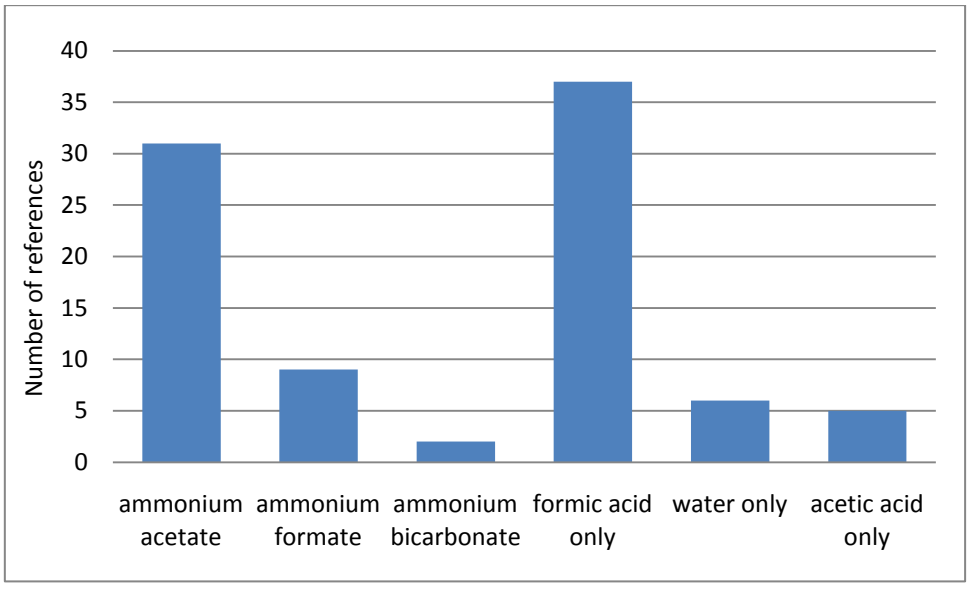


Figure 1-10; Journal of Chromatography B 878 Volumes 1 to 26: aqueous mobile phase composition

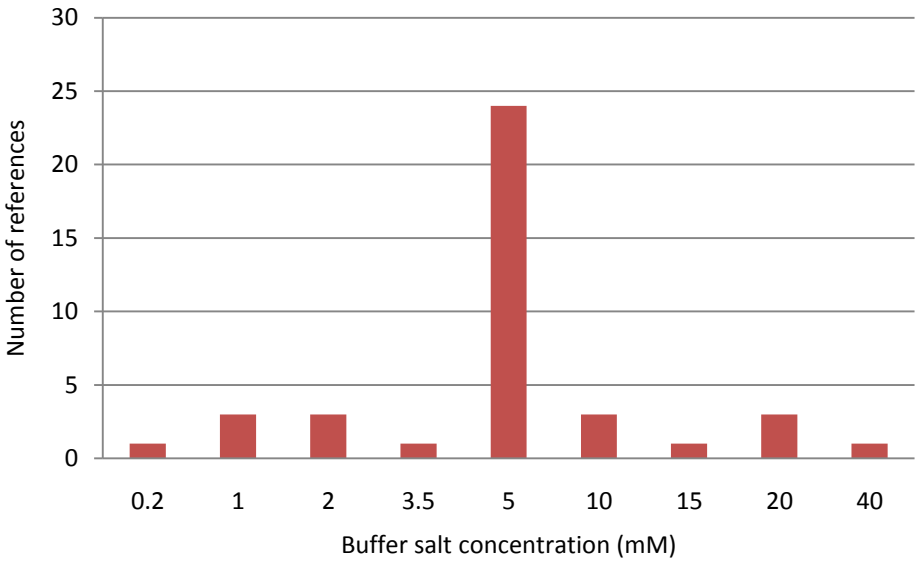


Figure 1-11; Journal of Chromatography B 878 volumes 1 to 26: aqueous mobile phase salt molar concentration

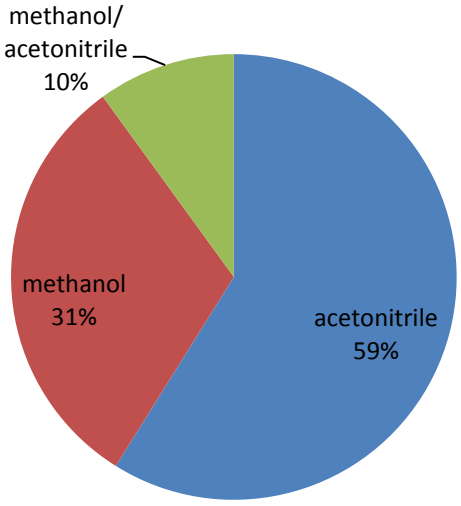


Figure 1-12; Journal of Chromatography B 878 volumes 1 to 26: organic mobile phase composition

1.3.5 Mass Spectrometric Quantification

Mass spectrometry as a quantitative detection device enables the derivation of a signal from compounds with the same mass to charge properties of the analyte of interest. It is therefore a very selective detection tool and enables the achievement of very low limits of quantification and removes the extent of chromatographic resolution from co-eluents necessary to define a clearly integratable chromatographic peak. Common constituents of the mass spectrometer are an inlet and ionisation device where analytes eluting from the chromatography column are transformed into gas phase ions at atmospheric pressure and then diverted via a series of lenses into a mass separation device. Here, ions are selected by

mass to separate ions with the mass of the target molecule and further fragmented in a collision cell before selection of a product ion, and transfer to an electron or photomultiplier tube to generate a signal proportional to the amount of ions present.

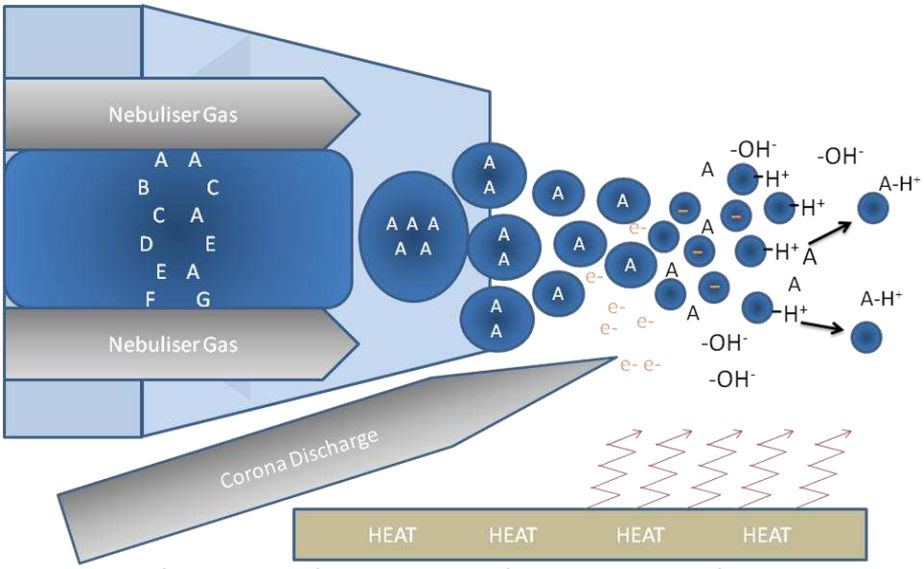
Ionisation source

The function of the ionisation source is to efficiently transfer the analyte in the liquid phase at atmospheric pressure to its ionised form in the gas phase. There are a number of ionisation source types available, but the ones used for pharmaceutical bioanalysis are those generally considered as the 'soft' ionisation techniques of atmospheric pressure chemical ionisation (APCI), electrospray ionisation (ESI) and occasionally, atmospheric pressure photo-ionisation (APPI). The 'soft' terminology describes the fact that they can be optimised to ionise compounds without causing further fragmentation in the ionisation source which may be induced by higher energy techniques such as electron bombardment. This ensures that the maximum mass of analyte is transferred to the mass analyser to maintain sensitivity.[94] The ionisation sources are suited to the typical mass range of small molecule pharmaceuticals 200 to 800 Da, and to labile metabolite compounds which may degrade in source.

In APCI, the eluent enters the source and is dried rapidly to remove residual solvent vapour by a heated nebuliser containing a carrier gas. The stream of volatilised solvent containing the analyte molecules are exposed to electrons generated by a corona discharge needle. The electrons cause the ionisation of solvent vapour molecules which transfer charge to the neutral analyte via proton transfer reactions. In APPI, the corona discharge needle emitting electrons is replaced by a photon source and the transfer reactions are promoted by the addition of additional dopant solvents, eg toluene. In both cases, ionisation is achieved while the analyte molecules are in the gas phase. A schematic representation of the APCI source is illustrated in Figure 1-13.

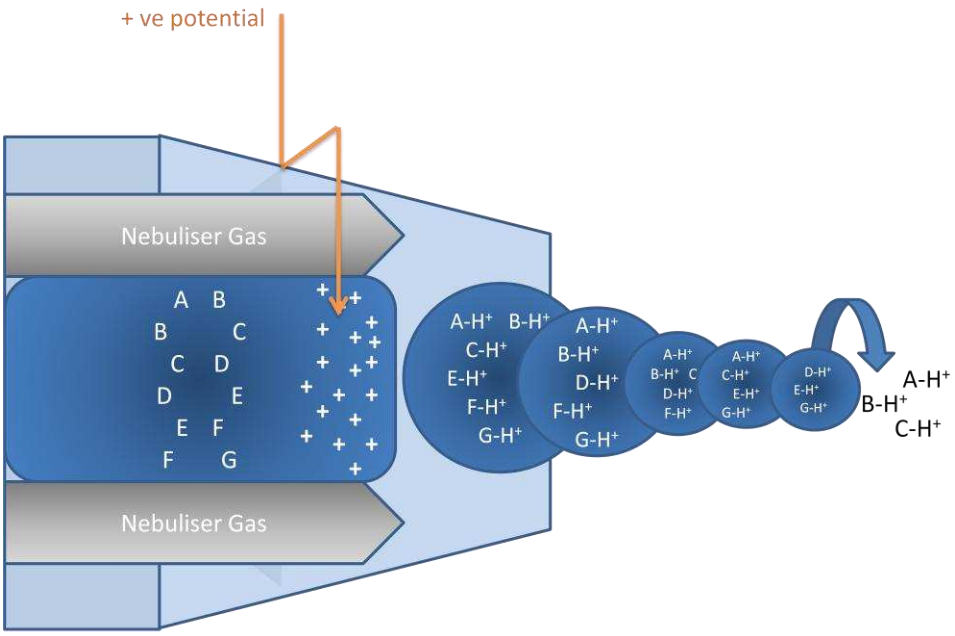
In ESI, before entering a desolvation chamber, the eluent passes through a capillary where a high electric potential is applied. The applied potential infers a charged surface and spreads out the droplets which form as they are nebulised in the presence of a desolvation gas and

increased temperature. The ionisation of analyte molecules occurs by the process of ion evaporation where as the charged droplets reduce in size due to desolvation, the charge density increases to the point where the field gradient is so large that 'coulombic explosion' occurs and charged analyte molecules are ejected into the gas phase. A schematic representation of the ESI source is illustrated in Figure 1-14.



HPLC eluent → Nebulisation → Corona discharge → chemical ionisation → proton exchange

Figure 1-13; Schematic atmospheric pressure chemical ionisation source



HPLC eluent → Nebulisation → → → Ion evaporation → → → → Coulombic Explosion

Figure 1-14; Schematic electrospray ionisation source

Mass selection

Of the many mass analysers available, the most frequently applied to quantitative LC-MS contain at least one quadrupole for the selection of the base peak. This quadrupole may be in-line with a second quadrupole where collision reactions may take place to induce fragmentation and a further quadrupole to select a fragment ion which can be used to generate a quantitative signal. Hybrid instruments containing a quadrupole and an alternative mass analyser such as an ion-trap or time-of-flight may be used to infer greater mass accuracy or structural information, by respectively allowing multiple fragmentation and mass selection reactions, or using an extended ion flight tube to increase mass resolution. The majority of reported methods for quantitative pharmaceutical bioanalysis in recent years; however, have been achieved using triple quadrupole mass analysers. In all cases, separation of ions by mass is achieved by generating an ion beam in a vacuum and adjusting the applied field to affect the stability of ions in the beam using direct current or radio frequency in a way which is proportional to their mass to charge ratio. The quadrupole is constructed with four symmetrical parallel rods through which oscillating radiofrequency (RF) fields are applied through diagonally opposing rods together with direct current (DC). The construction of the quadrupole is shown in Figure 1-15 showing the stable trajectory of an ion travelling through the quadrupole.

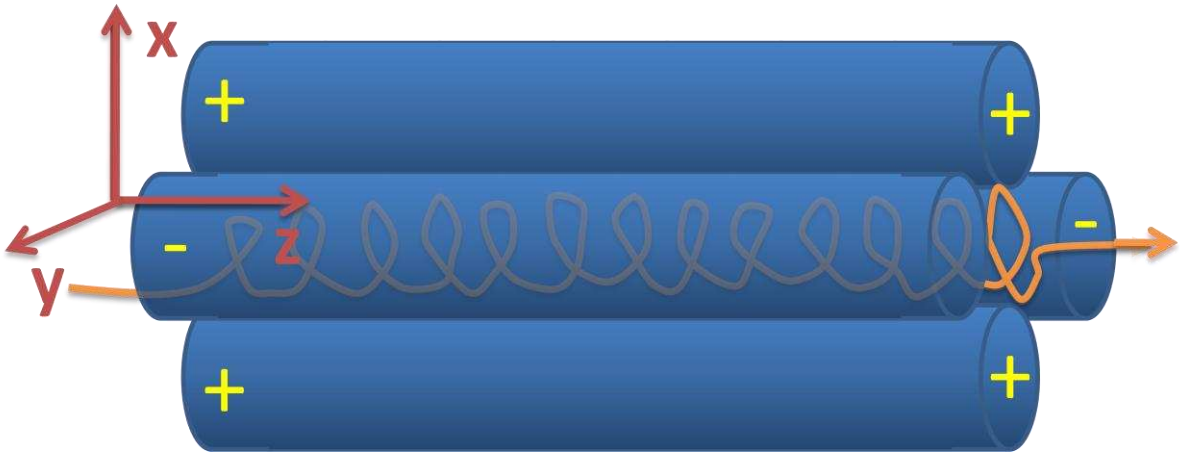


Figure 1-15; Diagrammatic representation the quadrupole showing opposing (oscillating) RF values linked diagonally. The orange line represents an ion travelling through the quadrupole in direction z, accelerated by DC and stabilised in the x-y axis by the oscillating RF frequencies

For each mass and charge, an ion with a trajectory in the z axis is stable in the x-y plane at a specific combination of RF potential and DC amplitude.

Detection device

The signal is generated when the filtered ion arrives at an electron multiplier. This consists of a horn shaped open tubular continuous dynode under vacuum coated with a semiconducting material such as lead oxide with a high negative potential at the entrance and ground state at the collector at the end of the horn.[95] Positively charged ions collide with the surface to release electrons which in turn are converted to secondary electrons during multiple collisions as the electrons are draw towards the ground state collector providing signal amplification. In some instruments, photo multipliers are used in place of electron multiplier tubes.

2 Bioanalytical Matrix Effects

2.1 Ionisation Enhancement and Suppression

Suppression (or inversely enhancement) of ionisation occurs when the efficiency of the ionisation process is either reduced or augmented by the presence of co-eluting species which either facilitate or inhibit charge transfer to the target analyte. As such, suppression and enhancement of ionisation processes are relative phenomena and it is generally accepted that some degree of suppression or enhancement are inevitable. The effects become critical when ionisation efficiency of the target analyte differs significantly between the test sample and calibrant samples giving rise to quantitative assay bias which is attributed to such 'matrix effects'. [96] The effect of uncontrolled ionisation suppression can therefore give rise to a greater signal response from unknown samples causing deviations in precision and accuracy not just loss of absolute sensitivity. [97]

From a regulatory perspective, matrix effects must be evaluated and controlled so that appropriate steps are taken to ensure the lack of matrix effects throughout the application of the method. [98] Routinely, this means that in validation, multiple different batches of control samples from the target matrix are analysed to verify that quantitative bias remains within defined limits. While these phenomena can occur in all atmospheric pressure ionisation sources, their impact is greatest during the electrospray process. [99] Pharmaceutical bioanalytical assays are generally standardised by using stable isotope labelled internal standards so within matrix ionisation effects are compensated.

Although matrix effects are defined relatively as suppression or enhancement, mechanistically, the phenomena are simply defined in terms of suppression of a theoretically complete ionisation process. In this case, suppression is caused by the action of species co-eluting with the analyte to prevent the analyte from forming ions in the gas phase. The mechanism of ionisation suppression is not clearly defined, a number of models have been proposed, and these may potentially influence the system simultaneously. [100, 101] Mechanisms of ionisation suppression were reviewed by Jessome and Volmer [100] and discussed by van Eeckhaut et al. [101] In electrospray ionisation sources, the net effect is that

analytes are prevented from transforming to gas phase ions in the condensed phase where electrospray droplets form. The following effects are presented as proposed mechanisms.

1. **Limitation of charge:** competition effects where co-eluting species ionise more efficiently, therefore preferentially to the target analyte which remains partly unionised. This is analogous to simply overloading the source with analytes to be ionised, leading to a loss of linearity at high concentration, biased towards more efficiently ionised analytes. [100, 102]
2. **Droplet surface properties:** co-eluents reduce the volatility of the electrospray droplet and increase its viscosity preventing ions from overcoming surface tension to enter the gas phase. [100, 103]
3. **Ion pairing effects:** cause neutralisation and co-precipitation of the charged analyte. [104]

Suppression effects have been identified as greatest when ionic species are present, [105] correlation was also made by King et al. [99] between the effectiveness of the extraction technique in eliminating non-volatile components with the relative reduction in matrix effects caused by ionisation suppression. Simple deproteinisation which results in the greatest proportion of non-volatile components remaining in the sample yielded a greater tendency to ionisation suppression than liquid-liquid extraction which optimally retains the more non-polar and volatile components. In general terms, liquid-liquid extraction is not universally applicable to small molecule bioanalysis due to the varying physicochemical properties of the target analytes. In the context of high throughput bioanalysis, throughput may be reduced by the requirements to carry out multiple solvent transfer steps increasing sample preparation time as well as resulting in an incompatible injection solvent for RP-HPLC. Extraction via SPE encompasses a broader range of analyte polarities and can readily be automated as earlier described (section 1.3.2). Karst and Liesener determined that effective separation on-line extraction columns such as Turboflow could be used to minimise ionisation suppression while maintaining sample throughput. [106]

2.2 Assessment of Matrix Effect

A quantitative assessment of ionisation efficiency and its contribution to process efficiency can be made by comparing the peak area responses of *(A)* the drug and its internal standard in post extracted samples, *(B)* samples prepared by addition of standards to extracted blank matrix, and *(C)* samples of the same concentration in unextracted solvents injected directly into the LC-MS instrument. [104] Extraction efficiency is determined by comparing A with B, ionisation efficiency, B with C, and process efficiency, A with C. The limitations of this method in in-line analysis methods are that *(i)*, plasma samples are injected into aqueous extraction buffers where solubilisation is achieved by plasma protein binding whereas injection of drug standards in solvents may cause loss of solubilisation, non-specific binding or solvent mediated effects on chromatographic retention on the extraction sorbent. Furthermore *(ii),* due to the composition of the analytical system, it is not possible to add drug standards to an extracted control sample without influencing the extraction. As a consequence, the ionisation efficiency and degree of suppression determined by this protocol applied to in-line extraction methods is only achievable as a gross process efficiency result including extraction efficiency.

A second and more versatile method of assessing relative ionisation efficiency in the presence of all co-eluents as a function of chromatographic retention time may be achieved by continuous post column infusion [107] as represented by Figure 2-1. In this method, a syringe pump is used to deliver a constant infusion of drug standard in an appropriate solvent into the mobile phase via a tee piece after the column while the mass spectrometer monitors the selected transition for the drug to generate a chromatographic baseline with a relatively high background caused by the constant ionisation and detection of the target analyte. Injection of a sample of blank matrix prepared by the test method under investigation is then acquired, and the potential of eluting species to enhance or suppress the ionisation mechanism of the target analyte are presented as peaks or troughs, respectively in the baseline. Repeating the same experiment but without the post column infusion and monitoring using full scan can then assist in identifying the compounds responsible for inducing suppression.

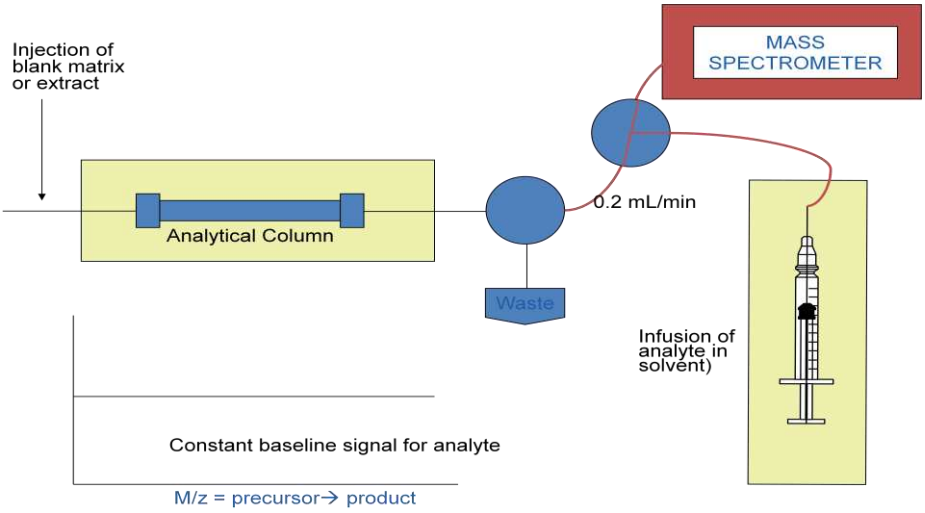


Figure 2-1; Schematic diagram of post column infusion

In bioanalytical assays, the complexity of the sample analysed means that suppressing species may arise by salts, [108] plasticisers, [109] mobile phase components, [103, 110] formulation components, [111- 113] concomitant medications, [114] and most critically endogenous components. [107, 115, 116] Importantly, while mutual ionisation enhancement/suppression of analyte and internal standard have been reported, [117, 118] the potential of co-eluting species to induce ionisation suppression is not necessarily related to structural or mass similarities to the analyte other than those which behave with similar chromatographic properties.

2.3 Phospholipid Biochemistry

A major endogenous source of ionisation suppression arises from phospholipids present in plasma samples. These polar lipids are diverse and abundant in plasma samples.

2.3.1 Mammalian Lipid Distribution

Lipids form the most diverse and abundant chemical class in biological organisms. The general chemistry encompasses fats, oils, some vitamins and hormones and form most of the non-protein components of cell membranes. They are formed by condensation of glycerol and fatty acids (Figure 2-2). [119]

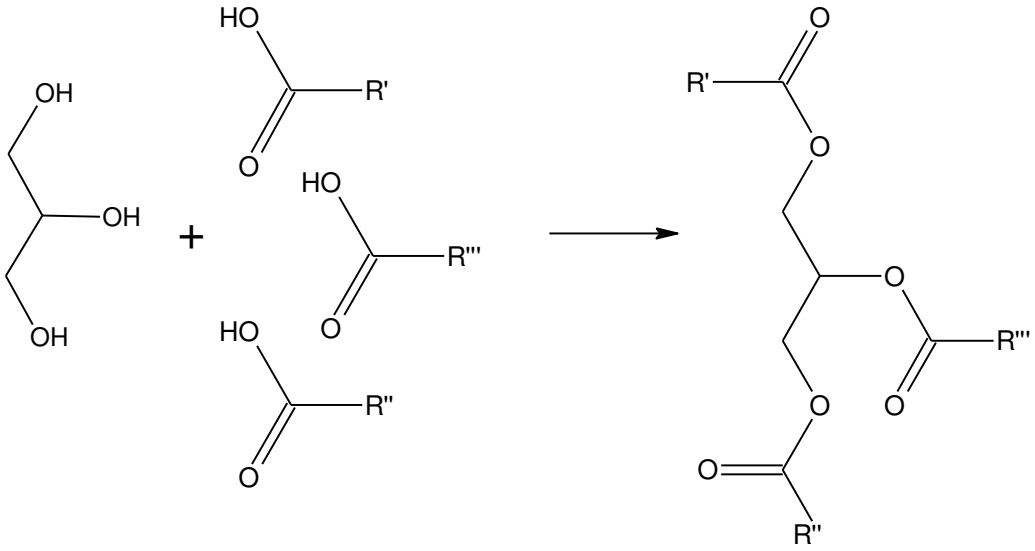


Figure 2-2; Formation of triglyceride lipids

Biologically abundant fatty acids are predominantly C16 and C18, almost half of biological fatty acids in animal lipids are unsaturated or polyunsaturated containing up to 3 double bonds. [119] The simple triglyceride represented by Figure 2-2 (tristearoylglycerol), the constituents of adipose tissue is the most abundant lipid type in the body, used primarily as an energy store. The normal human fat content is 21% in males and 28% in females. [119] Some abundant fatty acids are shown in Figure 2-3. The first double bond occurs between the C9 and C10 position from the carboxylic acid group in a fatty acid and in polyunsaturated fatty acids; double bonds occur every third carbon atom, always taking up the *cis* conformation. [119] The chain bending this infers on the fatty acid when substituted into lipids increases fluidity with polyunsaturation which is an important physicochemical property in biological membrane function. There are over 1000 known naturally occurring fatty acids although less than 50 are common. [120]

2.1 Membrane Lipids

Membrane lipids take the form of glycerophospholipids with the general chemistry indicated in Figure 2-4. Here R' and R'' are fatty acids which infer the diversity of molecular species and functionality to the biological membrane. Substituent X is the functional group which classifies the glycerophospholipid (Table 2-1).

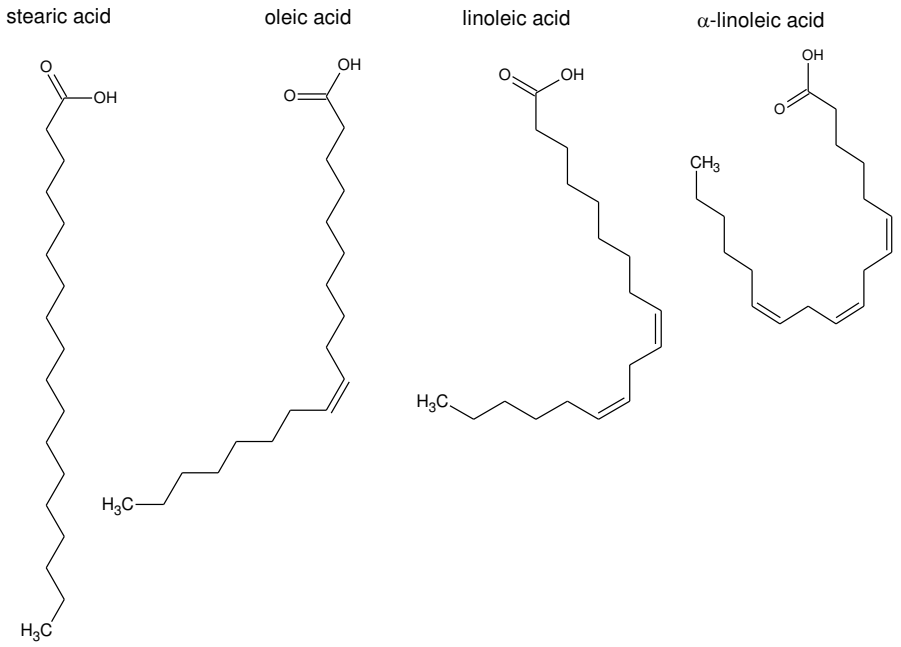


Figure 2-3; Some C18 fatty acids showing effect of degree of unsaturation on conformation

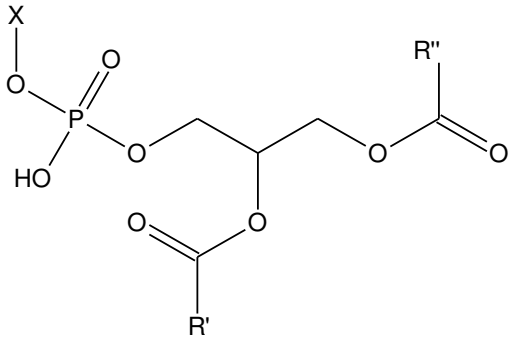


Figure 2-4; General chemistry of phospholipids

The highly surfactant nature of these compounds containing phosphodiester headgroup in place of one of the triglyceride fatty acid groups (with polar phosphoryl 'heads' and long nonpolar aliphatic 'tails') facilitate the aggregation into bilayers and micelles at low critical micellar concentrations of nano- or micromolar rather than millimolar concentrations observed in domestic surfactants such as sodium dodecyl sulphate. [121]

The nature of glycerophospholipids is highly diverse with 300 species existing in the human erythrocyte membrane alone. Biologically, in addition to barrier functions, phospholipids within membranes are responsible for solvation and modulation of membrane proteins, and their composition and location throughout the cell membrane is bioactively driven to be

complex and asymmetric across the membranes, altering in response to biological factors. They can also be involved in biosynthetic mechanisms and cell signalling. [122, 123] The field of lipidomics has arisen to associate the distribution and regulation of lipid species to determine functional biomarkers of disease state. [124, 125] Phospholipids have been identified as varying with changes in conditional states for example renal failure, [126] cancer [127] or leukaemia [128] and may serve as biomarkers of disease. It is clear that phospholipids are highly diverse and variable so their variable distribution between animal species and potentially between human groups is feasible. While these lipids form cell membranes, they are transported in plasma as excretory products.

2.2 Phospholipid Classes and Nomenclature

Substitution of the X group in the general glycerophospholipid chemistry in Figure 2-4 identifies the major phospholipid classes in mammalian cell membranes summarised in Table 2-1. While the phospholipid groups and their fatty acid residues can be described with both IUPAC and trivial nomenclature, they are most commonly described in the literature for simplicity by the carbon and bond number of each bonded fatty acid and a fatty acid nomenclature which identifies the position of the first double bond with respect to the terminal methyl group. [123, 120] For example, linoleic acid (Figure 2-3) may systematically be named as 9,12-octadecadienoic acid or alternatively as 18:2 (n-6) fatty acid.

Lipid	**Phosphodiester functional group**	**Percentage found in Human Erythrocyte [129]**	**Percentage found in Human Myelin [129]**
Phosphatidic acid	-H	1.5	0.5
Phosphotidyl choline*	$-CH_2CH_2N(CH_3)_3^+$	19	10
Phosphotidyl ethanolamine*	$-CH_2CH_2NH_3^+$	18	20
Phosphotidyl inositol*		1	1
Phosphotidyl serine	$-CH_2CH(NH_3^+)COO^-$	8.5	8.5
Phosphotidyl glycerol	$-CH_2CH(OH)CH_2OH$	0	0
Sphingelomyelin	$-CH_2CH_2N(CH_3)_3^+$ Additionally, *sn2* is substituted with $-NH(C=O)-(CH_2)_nCH_3$	17.5	8.5
Cholesterol		*10*	*26*
Glycolipids		*25*	*26*

* *Lyso*-phosphatidyl~ species are also identified where the acyl residue on one of the 2 available glycol groups is replaced by hydrogen so that the lipid has only one non-polar tail.

Table 2-1; Cell membrane phospholipid classes found in mammals

2.3 Mass Spectrometric Analysis of Phospholipids

Mass spectrometric determination of the major membrane phospholipid groups has been described by Millne et al. [122] The species that can be ionised in either positive or negative ion electrospray ionisation are identified in Table 2-2. The method used to identify the phosphotidylcholine species is by monitoring precursors of m/z = 184. The characteristic detection of each phospholipid species are summarised in Table 2-2

Analysis of the LC-MS chromatograph obtained by the above scanning mode allows the speciation of each group of phospholipids while the mass of each compound can be determined.

Species	**Ionisation mode** [121]	**Scanning mode**	**Headgroup specific ion** [122]	**Origin**
Phosphotidyl choline*	+	Precursor ion scan	184	Common fragment of $H_2PO_4CH_2CH_2N^+(CH_3)_3$
Phosphotidyl ethanolamine*	+	Neutral loss scan	141	Loss of $H_2PO_4CH_2CH_2NH_3$
Phosphotidyl serine*	+	Neutral loss scan	185	Loss of $H_2PO_4CH_2CH(NH_3^+)COO^-$
Sphingelomyelin	+	Precursor ion scan	184	Common fragment of $H_2PO_4CH_2CH_2N^+(CH_3)_3$
Phosphotidyl inositol	-	Precursor ion scan	241	Loss of $H_2PO_4C_6H_{11}O_5$ loss of H and loss of H_2O

* *Lyso*-phosphatidyl~ species are also identified where the acyl residue on one of the 2 available glycol groups is replaced by hydrogen so that the lipid has only one non-polar tail.

Table 2-2; Ionisation and common fragmentation of major mammalian phospholipid classes

3 Analysis of Samples from Dry Blood Spots

3.1 Principal and Applications

Collection of blood samples on filter paper was first described by Dr Robert Guthrie who demonstrated a technique of sampling from neonates to enable early screening for phenylketonuria (PKU) by measuring phenylalanine in dried blood spots (DBS). [130, 131] The procedure known as the Guthrie test is routinely carried out on newborns in hospitals globally where blood is drawn from a puncture in the heel within the first week of birth and taken on to screen against indicators of an array of congenital disorders such as congenital hyperthyroidism (CHD), sickle cell disorders (SCD), cystic fibrosis (CF), medium chain acyl-coA dehydrogenase deficiency (MCADD). [132]

The samples first taken using Dr Guthrie's method were analysed by bacterial inhibition assays, where the presence of phenylalanine leached from the paper into an agar disk mediated bacterial cell growth that had been chemically inhibited. With the initiation of mass screening which followed the introduction of the neonatal heel prick, an array of additional diseases screened shortly after birth were added to the tests. The main biochemical disease groups tested were organic acidemias and fatty acid oxidation disorders. [133] The presence of multiple screened conditions each with threshold limits identified for multiple diagnostic fatty or amino acids meant that specific bacterial inhibition assays were unable to meet the throughput requirements. GC-MS and LC-MS assays enabled the direct monitoring of markers such as arginosuccinic acid, while phenylalanine could be measured simultaneously with tyrosine indicatively as a ratio. Quantitative/semi quantitative HPLC-ESI-MS detection of DBS samples was able to facilitate sensitive, robust and high throughput neonatal screening analysis amounting to six 96-well plates per day. [134] A strategy for the comprehensive analysis of a battery of amino acids using a multiple gradient approach to adequately analyse all relevant acids without the need for derivatisation was described using HPLC-MS/MS. [134]

The application of DBS-LC-MS analysis was logically applied to therapeutic monitoring of exogenous compounds where drug concentrations in blood are analysed to verify therapeutic exposure to the drug substance. This is critically important where patient specific dosage is optimised based on drug exposure to achieve therapeutic concentrations

in the body while minimising side effects where the therapeutic index is narrow. [135, 136] It is also important in aggressive therapies where unwanted side effects are unavoidable and increase the tendency towards non-compliance. [137, 138]

A key advantage of dried blood spot sampling over analysis from whole blood or plasma liquid samples is that samples may be drawn outside of a laboratory or clinic and ideally in a home setting. For patients using the immunosuppressive drug tacrolimus this reduces return visits to hospital following transplant surgery since capillary blood samples can be taken onto a sampling card following finger puncture using a disposable lancet and without the presence of an expert phlebotomist then mailed back to the clinic for analysis. [139] This is important for patient convenience and therefore increases compliance with sampling; however the technique is also important in hospital settings which lack the equipment such as centrifuges, freezers and frozen transport which would be necessary to take plasma samples. This is the case for many studies taking place in remote locations or developing countries where epidemiological studies are conducted. Drug development studies involving anti-malarial treatments are typically monitored by DBS for this reason. [140- 143]

Significantly, the volumes of blood required for DBS analysis are far lower than those required for plasma sampling. Firstly since the total volume of blood drawn is taken into the sample for DBS while the red blood cell portion is removed by centrifugation to yield plasma meaning that the blood sample volume required per unit plasma volume is almost double. Sampling into appropriate volume blood collection tubes and transferring to plasma tubes limits the lower volume of blood to hundreds of microlitres while spotting onto filter paper can be completed in a single drop of tens of microlitres. This factor meant that DBS could be applied to rudimentary pharmacokinetic evaluation in discovery studies in rodents as described by Bateman and Beaudette. [144] The total volume involved meant that serial bleeding of rodent specials could be used instead of destructive, or stratified serial bleeding [145] (see Chapter 4) reducing total animal number and also improving data by reducing inter-animal variability.

The use of DBS for fully quantitative pharmacokinetic studies was first described by Spooner et al. in 2008 [146] following a pilot study which monitored paracetamol in dogs by HPLC-MS/MS using samples of blood dried onto paper. A method was demonstrated where

samples were cut from a dried blood spot and reconstituted into a methanolic solution of deuterated analogue internal standard. The resulting extract was analysed by HPLC-MS/MS with a lower limit of quantification of 100 ng/mL. The published assay validation data and application to analyse *ex-vivo* samples from an exploratory pharmacokinetic study demonstrated that the technique could be applied to support studies with GLP regulatory compliance. The method was later applied to analyse samples from a clinical study. [147] The validation methodology was subsequently applied to a number of further analytes and incorporated into a drug development study strategy. [148, 149]

3.2 Paper Media

Traditionally, for neonatal screening, diagnostic and analytical applications, blood samples were spotted onto uniform untreated cellulose based paper, often described as 'filter paper' although the specification of the paper media has been dedicated to blood sample storage for many years. The application of dried samples to paper was importantly utilised in nucleic acid analytical applications to collect samples for DNA fingerprinting, genetic analysis or epidemiological analysis by RNA/DNA analysis of microbiological or parasitic infection.

While dried blood spot sampling is used for the collection of epidemiological nucleic acid analysis of blood borne pathogens and DNA fingerprinting, the most common and least invasive technique of sample matrix application to the paper for forensic identification purposes is buccal swab sampling. Here, small amounts of tissue are brushed from the inner cheek membrane of the subject and applied to the paper. Although blood cells lyse on drying to release cell contents, additional workup is required to release nucleic acids from other tissue cells. A modified paper type was designed to facilitate the extraction of DNA from cells applied to paper media by impregnation with chemicals to promote rapid cell lysis to release nucleic acids which are then immobilised within the fibres of the cellulose paper for storage and transport, while further impregnated chemicals promoted virus and enzyme activation and nucleic acid stabilisation. As well as availability for immediate downstream Polymerase Chain Reaction (PCR) analysis, the stability inferred to the sample by this process enables storage of archive samples for many years. [150] FTA® paper is now routinely used for forensic and epidemiological nucleic acid sampling. [151-153] This impregnated media was further modified to produce a second generation paper type which enabled nucleic

acids to be simply desorbed with an aqueous wash, considerably simplifying the nucleic acid extraction process and is marketed as FTA elute®. [154]

Currently, FTA® and FTA elute® supplied by Whatman are the only 2 chemically modified paper types available while a number of untreated paper types have been used for DBS applications with grade 31ETF and grade 903 papers supplied by Whatman (GE medical)[A] and Ahlstrom 226 supplied by ID Biological[B] are the predominant papers with sufficient uniformity for quantitative analysis. Recently, blood collection for pharmacokinetic analysis has used impregnated FTA®/FTA elute® papers to combine the advantages of rapid virus and enzyme inactivation with the alternative elution characteristics of 2 different modified paper types with the alternative elution selectivity from pure cellulose paper. [146- 148] The chemically modified papers are not without limitations since loading volumes are limited by the capacity of the impregnated chemicals. The advantages and disadvantages to the use of modified and pure cellulose DBS paper are summarised in Table 3-1.

3.3 Extraction Methodology for Small Molecules

Solvents

The first step for all analyses involving extraction from the sampling paper is to remove an aliquot of the paper by punching out a circle of paper containing sample into an extraction tube or microtitre plate. In the literature these vary from 3 to 8 mm diameter and can be cut by hand using a Harris unicore punch or equivalent or dedicated robotic punching equipment. The method used to extract pharmaceutical molecules from paper varies and is compound specific. Tawa et al. utilised 0.5 *M* Na_2HPO_4 (pH 8.7) for the elution of aminoglycoside antibiotics. [136] Allanson et al. used acidic buffering of rifampicin dried blood samples on paper with 0.02 *M* ammonium acetate (pH 4.0) followed by extraction into methanol and subsequent evaporation and reconstitution in injection solvent. [155] Liquid-liquid extraction of buffered punches into larger volumes of aqueous immiscible solvents such as methyl tert-butyl ether were reported prior to evaporation and reconstitution [148] with the ability to selectively extract and separate steroids and their glucuronide

[A] www.gehealthcare.com

[B] www.id-biological.com

metabolites between basic organic and aqueous layers prior to derivatisation. [156] Ideally, if sufficiently rapid extraction mass transfer is possible, the punched paper extract may simply be vortex mixed with an aqueous solvent mixture containing internal standard with removal of the paper punch to form the HPLC injection solvent. [138, 146, 157]

Paper type	Advantages	Disadvantages
1. Pre-treated; cellulose based, impregnated with patented chemicals to lyse cells, fix DNA, and enable DNA recovery. (FTA®, FTA elute®)[C]	• Documented to inactivate viruses • Demonstrated to rapidly inactivate plasma enzymes • Provides alternative recovery selectivity • Long term physical stability determined for the storage of genetic samples	• Currently 1 vendor. • Volume (spot size) limitations • Moisture sensitive • Distribution effects attributed to loading chemical treatments • May be difficult to store and handle, • May be light and temperature sensitive
2. Non treated paper; cellulose based, high purity paper. (Whatman 903, Whatman 31ETF[D], IDB226)	• Less expensive with multiple vendors • Large spots possible, evenly distributed • May be heated to accelerate drying (to be tested) • No additional chemicals present. • No distribution effects or volume limitations caused by chemical overloading or moisture sensitivity (to be verified)	• Does not rapidly inactivate enzymes • Data on virus inactivation unavailable - historical evidence of regular mail shipment (US CDC permitted) • Less scope for alternative recovery selectivity • Some grades may not give equivalent uniformity

Table 3-1; Summary of DBS sampling paper properties

In instances where low extraction recoveries from the paper were limiting to assay sensitivity, novel methods of pre-treatment of filter papers have been reported. [158, 143] Van der Hijeden et al. [158] described impregnating Whatman 903 paper with acidic ammonium

[C] Also supplied as FTA® DMPK-A and FTA® DMPK-B respectively

[D] Also supplied as FTA® DMPK-C

acetate buffered pasteurised plasma protein solution which was applied to the paper and dried prior to blood sampling for analysis of the antimalarial everlimus. Blessborn et al. [143] pre-treated paper with tartaric acid for the collection of blood samples to analyse another antimalarial, lumefantrine. This was necessary for the large acidic molecule to disrupt the gradual ionic binding to the paper which had been observed to give rise to the phenomenon of reduced recovery following several days' storage. [143]

Extraction Protocol

Samples extracted from the paper matrix into solvents may be either injected directly for separation on an HPLC column, or cleaned up by solid phase extraction, either off-line [142,143,159] or on-line. [158, 139] Sonication time was investigated by Damen et al. [160] in the extraction of vincristine and actinomycin-D from paper into acetonitrile:methanol:water solvent; they found a standard 15 minute sonication step was adequate for this transfer [160] while longer sonication times and centrifugal filtration of the paper were required for the everolimus assay of van der Heijeden et al. [158] Beaudette and Bateman [144] employed a 2 hour incubation step of the paper in the extraction medium at 37°C with shaking. The duration of exposure to the extraction medium for dry blood spots was investigated by Koal et al. [137] who found that for extraction recovery increased with time between 20 seconds and 75 minutes of contact with the extraction reagent following vortex mixing with 1 of the 9 antiretrovirals tested amounting to a 50% increase in recovery. [137] Clearly, to optimise recovery from the punched blood spot when sensitivity is likely to be a limiting factor, the method utilised in the extraction process– shaking, sonication, heat must be optimised in addition to extraction medium chemistry. Liu et al. Introduced the term 'elution efficiency' to describe this. [161]

Factors Influencing Distribution and Recovery

One factor which may be significant in the recovery of analyte from the spot is water content. Water may arise from drying time of the spot following sampling or the ambient humidity of the card or dried spot storage. It is particularly significant for the chemically modified media of FTA® and FTA elute® which contain hygroscopic components in formulations and FTA elute® is designed to facilitate easy nucleic acid elution in aqueous reagents. As a result, it is imperative that samples are stored under desiccation prior to

extraction. Hoogstanders et al. [139] investigated the time to dry a typical 30 µL spot by weighing papers after timed ambient drying, and found that up to 18 hours, the mass of the spot was still falling due to sample drying, [139] however; Koal et al. [137] related drying time to extraction recovery and found there to be no significant difference after 2 hours for up to 5 days, [137] although it is notable that both work was done using untreated paper which is less sensitive to moisture.

An aspect with the potential to affect the distribution in paper is the blood sample rheology, particularly the effect of shear viscosity on the percolation through the paper matrix. The more viscous a blood sample is upon application to the paper, the more slowly it will percolate through the paper and therefore dry to form spots with smaller radii than spots of the same volume from a less viscous matrix. In consequence, the unit area of sample cut from the spot for analysis potentially contains a differing proportion of the applied blood sample. The significance of the extent of this affect on the analytical bias of the assay is subject to validation. The influence on anticoagulant [162] and clinical condition of the donor [163] on the shear viscosity of blood is well studied; Reinhart et al. investigated a range of commonly used blood anticoagulants and showed EDTA to result in the lowest blood viscosity. [164] Rosenblum [162] showed that elevation in the relative viscosity of blood samples taken from animals using citrate anticoagulant was initiated by red cell shrinkage caused by the anticoagulant. He related the action of each anticoagulant as those which induced shrinking of the erythrocyte (citrate and oxalate) and those with no effect on shape or size (heparin, EDTA) to the effect of the anticoagulant on the viscosity of blood.

Rosenblum also related alternative anticoagulants to changes in hematocrit; the proportion of blood volume occupied by red blood cells. [162] The hematocrit is known to have a proportional relationship to blood viscosity. [165] Mei et al. [166] related hematocrit volume with the amount of serum extracted from 6.0 mm punches cut from 100 µL blood spot samples across a range of hematocrit values between 30 and 70%. An increase in hematocrit corresponded to a decrease in serum content of the punched spot for 2 sources of unnamed untreated filter paper. [166] Holub et al. [167] studied the effect of hematocrit on recovery of acylcarnitines and amino acids from neonatal samples and found recoveries to increase proportionally to hematocrit.

The potential exists for distribution differences as the blood sample percolates through the paper media which have been compared to chromatographic effects. [166] In untreated paper it has been shown from neonatal samples that punches cut from the centre of the blood spot may give rise to differing recoveries to those cut from peripheral positions. [167] In treated papers the additional complexity of impregnated chemicals which may overload causing changes in the paper medium with volume spotted limit the spot size, while differing migration rates of components of the blood following contact lysis give rise to visibly unevenly distributed samples with serum rings forming around the lysed erythrocyte centre. [147] Ren et al. demonstrated the potential for deviation from homogeneity using autoradiography to image the distribution of ^{14}C labelled compounds added to blood and spotted to different paper types. [168] They also related storage environmental conditions to the extent of the effect in treated papers.

3.4 Safety Implications

The most significant hazard when handling biological fluids, particularly from human or primates is exposure to etiologic agents infecting the blood. Viruses such as HIV and particularly hepatitis B and C present a hazard in handling known positive or unscreened blood and plasma samples and have been transmitted as laboratory associated infections. [169] In the pharmaceutical industry, liquid samples are routinely handled in biosafety containment cabinets [170] in conjunction with appropriate personal protective equipment and following risk assessment.

Transportation of plasma is by frozen shipment, [171] packaged into polystyrene boxes containing dry ice which maintains the sample at -78°C to prevent purification and analyte instability. Since many etiologic agents are still active under these conditions, the samples are subject to the International Air Transport Association (IATA) Dangerous Goods Regulations. Transport and handling of samples as dried blood spots however, presents a lower biological risk since many viruses such as HIV are rapidly inactivated on drying [172] while others such as Hepatitis B reduce in activity with time. [173] Where no data exists on viral inactivation on drying, the reduced sample volume and reduced possibility of ingestion into the bloodstream of material immobilised onto paper presents a lower infection risk, viruses loose infectivity due to disruption of their envelope on drying. [174, 175] As a result, the

International Air Transport Association (IATA) recommends that dried blood spot samples are not subject to the dangerous goods regulations. [176] Indeed, transport of samples from epidemiological investigations to study infection rates of Hepatitis C and HIV (generally by viral RNA analysis) have been routinely conducted using regular mail transport. [177- 179]

3.5 Ethical Considerations of DBS in Drug Development

The key advantage of DBS sampling from an ethical perspective is the ability to collect samples in very small volumes (a few drops of blood). For pharmacokinetic bioanalysis, developments in HPLC-MS/MS in recent decades have facilitated the achievement of analytical methods with sufficient sensitivity to achieve specified lower limits of quantification at low sample volumes. However, the practical volume limitations of blood withdrawal, plasma centrifugation and volumetric pipetting and the loss of sample residues on multiple surfaces meant that typical sample volumes remained in the order of hundreds of microlitres of whole blood. DBS provided Spooner et al. with a medium to obtain blood volumes of typically 15 µL, at least an order of magnitude less than those required for plasma analysis of pharmacokinetic study samples. [146- 148] Ethical considerations are at the core of the decision of Spooner's organisation, Glaxo SmithKline to replace plasma analysis with DBS for all feasible drug development programmes. Their approach is to implement DBS analysis when drug molecules enter development, since drug exposure will be defined as blood concentrations rather than plasma concentrations and differences in kinetics may arise from distribution into blood cells. The immediate contribution to the principals of 3Rs (see chapter 4) is the refinement of a less invasive sampling protocol. Small blood volumes withdrawn by sampling capillary via the lateral caudal vein of rats and mice is much easier and with less potential to cause stress. As occasionally practiced in human sample withdrawal, for the withdrawal of blood volumes required for plasma analysis, the animal is warmed to increase blood flow by gently comfortably increasing the temperature of the animal housing for a period of time before the sample is taken. Lovatt et al. [180] reported that this step may be eliminated for rat sampling and reduced for samples from mice. Critically, since less of the blood volume of the animal is taken for each sample, more sample time points may be taken, meaning that the number of animals used to generate the dataset may be reduced. Limiting inter-subject variability additionally improves data quality.

Evolution of drug development into clinical trials, the less invasive methodology is anticipated to improve patient recruitment since capillary blood sampling by finger prick is less objectionable to the patient or volunteer than venopuncture. As has been demonstrated by therapeutic monitoring reports, the method is consistent with late phase monitoring where samples may be taken at home without visiting a clinic to see a phlebotomist. This is most important where paediatric studies are conducted to adequately study pharmacokinetic characteristics of drug molecules in children.[181, 143] Such studies and their impact on the safety evaluation of drugs in the target population where pharmacokinetics may not have routinely been studied are promoted as a priority by pharmaceutical regulatory authorities.

4 Ethical use of Animals in Pharmaceutical Research

4.1 The use of Animals in Preclinical Safety Studies

Before new drug compounds can enter clinical development, where single low doses are administered to healthy human volunteers, extensive data on safety must be compiled by conducting essential studies into various elements of the physiological effects of the new medicine. This is not only essential in protecting the safety of volunteers who will be exposed to the new pharmaceutical compound before calculating the first dose, but is a legal prerequisite of the regulatory authorities before approval to initiate clinical trials and define the endpoint of subsequent dose escalation. [182, 183] Safety pharmacology and acute toxicity studies from single and repeat dosing supported by pharmacokinetic studies to define the dose relationship to adsorption distribution, metabolism and excretion of the molecule must be conducted. Within Sanofi-Aventis, every scientific protocol is reviewed by an ethics committee before approval with emphasis on animal welfare.

4.2 Blood Sampling Volumes and Study Samples

Blood samples are taken according to strict guidelines by qualified personnel under licence in the UK by the home office. [184, 185] For any animal, the maximum permissible non-terminal sampling volume is 15% of total blood volume in a 24 hour period. This is important for both animal welfare and the integrity of the study to ensure that any physiological effects of blood loss do not contribute to pharmacologic observations.

The sampling protocol adopted by the study design is dependent on the sampling frequency and volume requirements for analysis and the weight of the animal used to calculate blood volume. The protocols are in Table 4-1.

Serial sampling	Entire pharmacokinetic or toxicokinetic profile is sampled from an individual animal
Composite sampling	A single profile is derived from more than one animal, but each animal provides more than one sample
Terminal sampling	Animal is exsanguinated for a single sample

Table 4-1; Blood sampling protocols

The volume of plasma required for a pharmacokinetic sample analysis therefore influences the blood sampling protocols described above. For a pharmacokinetic study of 3 animals per sex per time point, and 6 time points required to define the pharmacokinetic parameters, the relationship between sample volume requirements and bleed protocol and consequently total animal number are calculated in Table 4-2.

Species	Plasma volume for analysis (µL)	Plasma volume requested (µL)	Blood volume required (µL)	Bleed Protocol	Blood volume per animal (µL)	Number of animals required
Rat	500	1500	3000	Terminal (1 time point per animal)	3000	36
Rat	200	600	1200	Composite (2 time points per animal)	2400	18
Rat	100	300	600	Composite (3 time points per animal)	1800	12
Rat	50	150	300	Serial (6 time points per animal)	1800	6

Table 4-2; Relationship between sampling volume, blood sampling protocol and animal number

From the data above, smaller analytical sample aliquots may facilitate more serial study design; reducing animal numbers used which additionally improves data quality by reducing inter-animal variability.

4.3 Blood Sampling and Control Matrix

To support the analysis of study specimens, analytical samples are routinely prepared from control plasma to construct calibration curves and quality control samples and frequently for the preparation of internal standard sample solutions. Due to the large dynamic range of concentrations analysed, from maximum post dosing concentrations to days after dosing

and across large dose ranges, it is often necessary to dilute the samples into the validated calibration concentration range using control plasma taken from untreated animals. The use of control plasma is matched by species, such that, for analysis of samples from a dog study, calibration and quality control samples, together with the working internal standard and dilutions must be prepared using dog plasma. This is the practice for analysis of samples subject to inspection for good laboratory practice [186] by the Food and Drug Administration (FDA) or Medicines and Health Regulatory Authority (MHRA) for US or UK markets, respectively.

In small rodent species, blood samples are removed under general anaesthesia to achieve total exsanguination of an anaesthetised animal which is therefore a destructive process and animals are necessarily destroyed to supply control plasma. Typical maximum blood volumes and estimated plasma attainable are shown in Table 4-3.

Species	Blood Volume (mL)
Rabbit	224
Rat (male)	25
Mouse	1.8

Table 4-3; Typical maximum blood volumes of laboratory rodents

Samples are usually diluted by adding 50 µL of sample to 500 µL of control plasma to enable sufficient pipetting precision and accuracy. For a typical rat toxicokinetic study, this might amount to 100 to 200 mL of control plasma.

4.4 Reduction Refinement and Replacement

Following recommendations of the House of Lords select committee on animals in scientific procedures 2001 report, the National Centre for the Reduction, Refinement and Replacement of Animals in Medical Experiments (NC3Rs) was formed to provide an ethical framework to experimentation using animals. [187] The mission of the centre is to reduce, refine and replace the use of animals in experimentation by working with scientific institutions and regulatory authorities and fostering innovative approaches to providing

scientifically valid alternatives to animal use without compromising protection of human health.

The principals of the 3R are supported by the major pharmaceutical companies and the Association of the British Pharmaceutical Industry. Sanofi-Aventis subscribe clearly to the 3Rs strategy and the fundamental importance of animal welfare and culture of animal care is central to the charter for the humane care and use of laboratory animals. [188]

4.5 Research Aims and Objectives

The aim of this project was to optimise an LC-MS/MS bioanalytical assay method to focus on ethical objectives of minimising blood volume requirement for sample analysis. It was proposed to investigate the substitution of animal plasma control matrix for plasma withdrawn from a larger, non-destructively bled species primarily to replace rodent plasma as control matrix for dilutions and calibrant sample preparation. While the use of surrogate matrices has been reported where matched matrices are unobtainable or frequently contaminated by the test item, [189, 190] extensive cross validation for ethical purposes has not been reported. Previous obstacles to cross calibration between species were investigated to identify the effects of endogenous biochemical components on the integrity of cross calibrated assay methodology. The main objective was therefore to demonstrate valid and unbiased analysis of a pharmaceutical sample in rodent plasma by extrapolation against control samples prepared in human plasma relative to matrix matched analysis.

The recently adopted technique of dried blood spot analysis of pharmaceuticals using blood as an exposure medium instead of plasma following extraction of the sample from paper matrix was also to be investigated with the object of facilitating volume reduction of sample requirements for analysis. This could then be translated into smaller animal sample volumes, leading to refined sampling of fewer animals.

The principal is also supported by regulatory agencies responsible for scrutinising the validity of methodology used in the conduct of analysis used to generate data for pharmaceutical safety studies. This was expressed by Beharry who stated her opinion that the UK Medicines and Healthcare Regulatory Agency (MHRA) are fully committed to encouraging the development of alternative methods and actively seek to influence

international harmonization initiatives to ensure the protection and welfare of animals and to minimise the numbers of animals used. [191]

5 Experimental

5.1 Reagents and Materials

5.1.1 Chemicals

All drug standards and stable isotope labelled internal standards were obtained from Sanofi-Aventis. Acetonitrile, methanol, propan-2-ol (HPLC grade) and dimethyl sulphoxide (reagent grade) were obtained from Fisher Scientific (Loughborough, UK). Formic acid (free acid 95% and ammonium salt, both ACS grade) and ammonium hydroxide were obtained from Sigma Aldrich (Dorset, UK). Ultra-pure water was generated using an Elga maxima water purifier (Marlow, UK) and verified as having maximum conductivity of 18.2 $M\Omega.cm^{-1}$ prior to use.

5.1.2 Biological Material

Pooled control human plasma screened and free from antibodies to HIV and Hepatitis B and C from blood drawn into lithium heparin, sodium citrate, sodium heparin and potassium EDTA tubes were obtained from Biochemed (Winchester, USA). Individual control plasma samples from Japanese volunteers drawn in EDTA anticoagulant were obtained from Sanofi-Aventis (Kawagoe, Japan); control plasma samples from individual human volunteers specially screened from Caucasian, Hispanic and African American origin fasted or lipemic were obtained from Biochemed (Winchester, USA). Fresh human whole blood was obtained from volunteers from Sanofi-Aventis (Alnwick, UK) drawn into potassium EDTA or lithium heparin vacutainers (Beckton and Dickson, UK). Whole rat blood was obtained from Sanofi-Aventis (Alnwick, UK).

Plasma samples obtained from individual animals were obtained by pooling intra-individual sample aliquots remaining from study samples taken from drug development programmes of other drug candidates following authorisation for sample disposal. Pooled blank plasma from rat, mouse, guinea pig, rabbit, dog and Cynamologous monkeys (Macaques) used to prepare control samples was obtained from Biochemed (Winchester USA). All liquid biological materials were handled in a class I biosafety cabinet (Astec-Microflow, Hants, UK). All plasma samples were stored frozen at -20°C in walk-in freezers (Thermo Scientific,

Basingstoke, UK) all liquid blood samples were stored under refrigeration at 4°C in refrigerators (Labcold, Basingstoke, UK).

5.1.3 Laboratory Consumable Equipment

Polypropylene microtitre injection plates (96-well) and injectable square sealing mat manufactured by Porvair were obtained from Fisher Scientific (Basingstoke, UK). Dried Blood Spot analysis paper Ahlstrom 226 was obtained from ID Biological (South Carolina, USA); Whatman FTA® and FTA Elute® DBS cards were obtained from GE Life Sciences (Amersham, UK).

5.1.4 Extraction and Analytical Columns

Large particle support columns were Turboflow® columns, C2, C8, C18 and Cyclone (reversed phase chromatography polymer), supplied by Thermo (Hemel Hempstead, UK). HPLC columns used were BetaBasic C8 50 x 3.0, 3 µm, supplied by Thermo (Hemel Hempstead, UK) and Gemini C18 50 x 3.0, 3 µm, supplied by Phenomenex (Macclesfield, UK).

5.2 Analytical Equipment

5.2.1 Sample Preparation and HPLC Separation

The HPLC-MS equipment (Figure 5-1) used was composed of an Agilent 1100 quaternary pump delivering mobile phase to the extraction column with an Agilent 1100 binary pump delivering mobile phase to the analytical column. Mobile phase flow paths were controlled to enable column switching using a valve interface module (VIM) unit, containing twin 6 port valves fitted with valco rotor seals, obtained from Thermo (Hemel Hempstead, UK). The two pumps were connected with the autosampler, VIM unit, extraction and analytical columns and the mass spectrometer using PEEK tubing as represented in Figure 5-2.

Mobile phase was degassed by in-line vacuum degasser (Agilent, Stockport, UK). Samples were injected using a PAL CTC autosampling microtitre plate injector fitted with a PEEK loop and Hamilton gastight syringe appropriate to the injection volume (Jaytee Biosciences, Kent, UK). Turboflow extraction columns were maintained at ambient temperature, while HPLC columns were thermostatically controlled using Phenomenex column ovens (Phenomenex,

Macclesfield, UK). HPLC autosampler sequences and HPLC gradient sequences and VIM were coordinated and data using Aria software (Thermo, Hemel Hempstead, UK).

5.2.2 Dried Blood Spot Cutting

Dried blood spot samples were stored in a Secador automatic desiccator supplied by Fisher Scientific (Loughborough, UK). Aliquots were punched for analysis using a BSD 6000 duet spot cuter (BSD Robotics, Queensland, Australia).

5.2.3 Mass Spectrometry

Mass spectrometry detection was using either a Waters Micromass Quattro Ultima or Waters Micromass Quattro Premier (Waters, Manchester, UK) triple Quadrupole mass spectrometer operating with an electrospray ionisation interface.

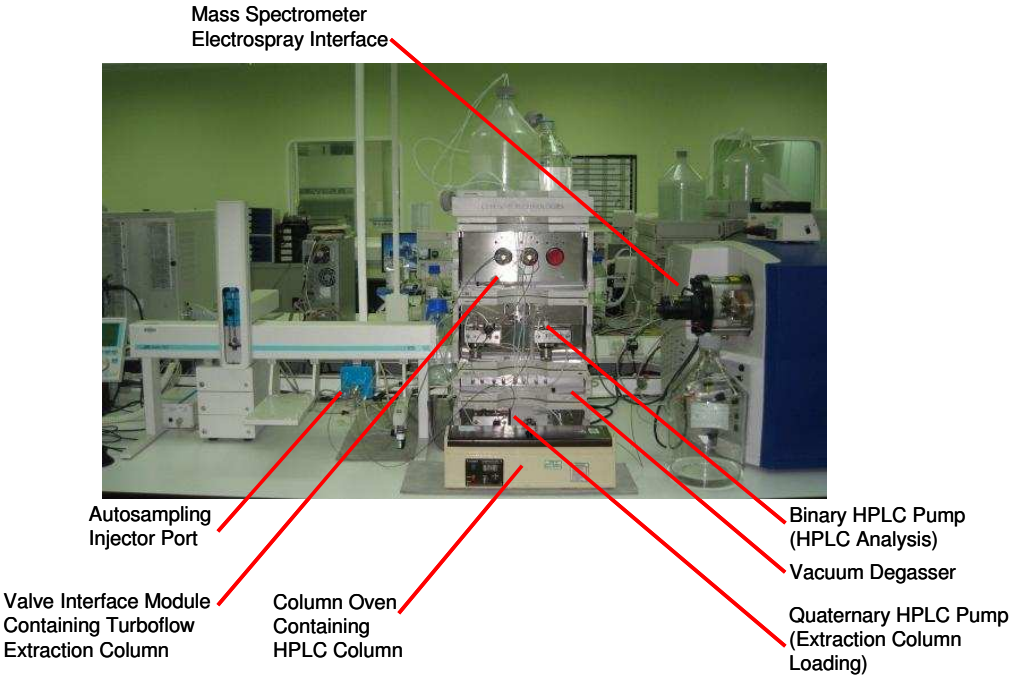


Figure 5-1; Turboflow HPLC-ESI-MS photograph

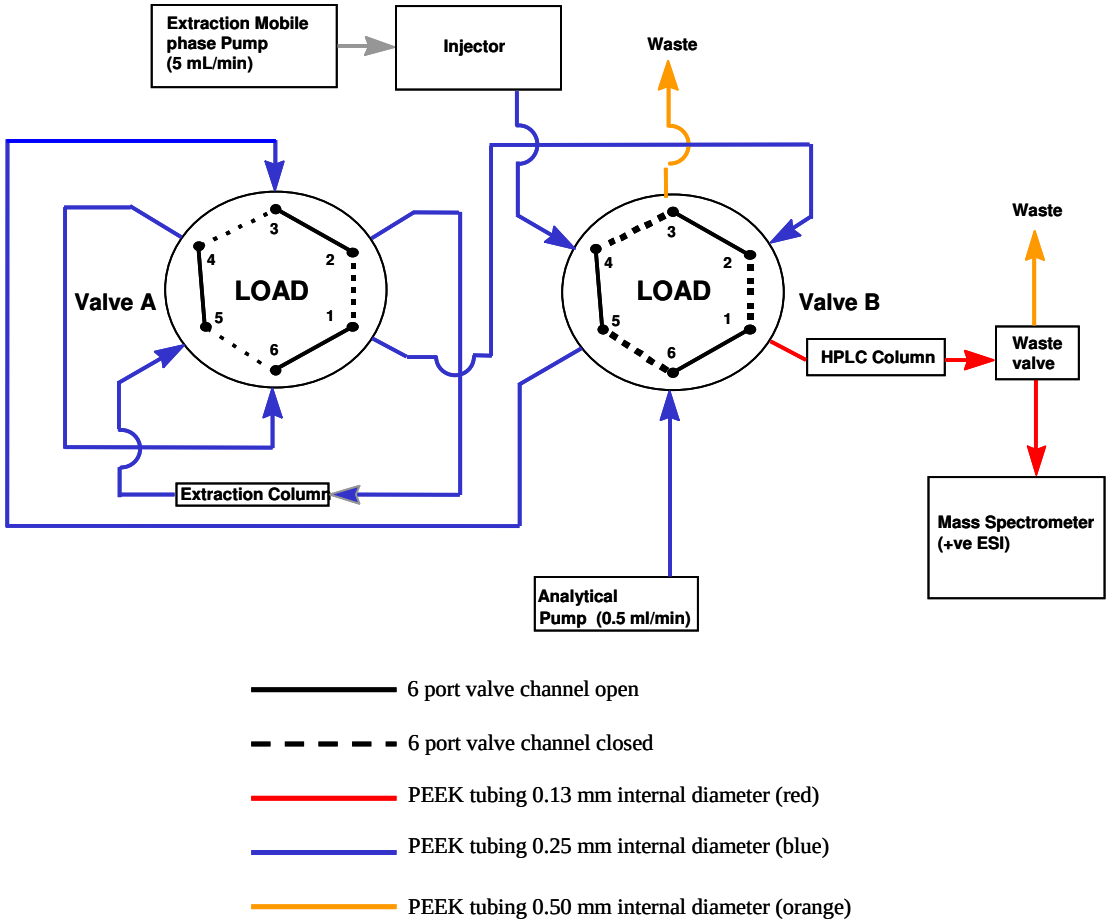


Figure 5-2; Tubing and valve interface

5.2.4 Computer Software

LC-MS data was acquired using Micromass Masslynx v1.4.1 (Waters, Manchester, UK). Peak integration was performed using Quanlynx v1.4.1 (Waters, Manchester, UK) and linear regression to obtain concentration data was performed using either Quanlynx or Watson LIMS (Thermo, Altrincham, UK).

Descriptive statistical analysis was carried out using either Microsoft Excel or Prism statistical software (GraphPad, La Jolla, CA, USA). Statistical design of experiments (DOE) analysis was done using MODDE 9 (Umetrics, Umea, Sweden), LC-MS data alignment was done using XCMS (Scripps Research Institute, La Jolla, CA, USA), multivariate analysis by principal component analysis (PCA) and partial least squares regression discriminative analysis (PLS-DA) was carried out using SIMCA P 11+ (Umetrics, Umea, Sweden). Physicochemical properties were calculated using log D suite software suite (ACD labs, Bracknell, UK).

6 Results and Discussion – SARX; Initial Work

6.1 Introduction

4-Chloro-3-[(3R)-5-chloro-1-(2,4-dimethyoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl]-N-ethyl-N-(3-pyridinylmethyl) benzamide (subsequently referred to as SARX; Figure 6-1) is a potent, selective oxytocin receptor antagonist which has been shown to reduce contractions induced by oxytocin. [192] The nonapeptide oxytocin is the most potent of the endogenous stimulants of uterine contractions and is expressed during the onset of preterm labour. Administration of the drug at the onset of labour has the potential to induce uterine quiescence and delay the gestational age of delivery to improve the neonatal outcome. The compound was in development by Sanofi-Aventis and at the start of this project, undergoing preclinical development studies while preparing to commence phase 1 (first in man) clinical trials.

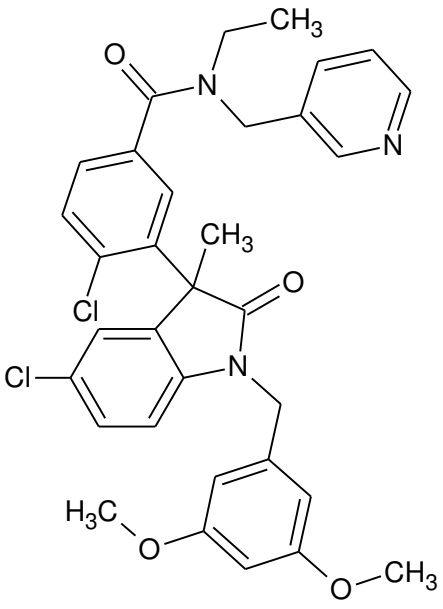


Figure 6-1; Structure of SARX

SARX is weakly basic with a molecular weight of 604.5 and a pKa of 4.72. The calculated Log P is 3.54 +/- 0.9 while the log D increases from approximately 1.5 at pH 2.5 to 3.5 at pH 6 and is unchanged at higher pH (Figure 6-2).

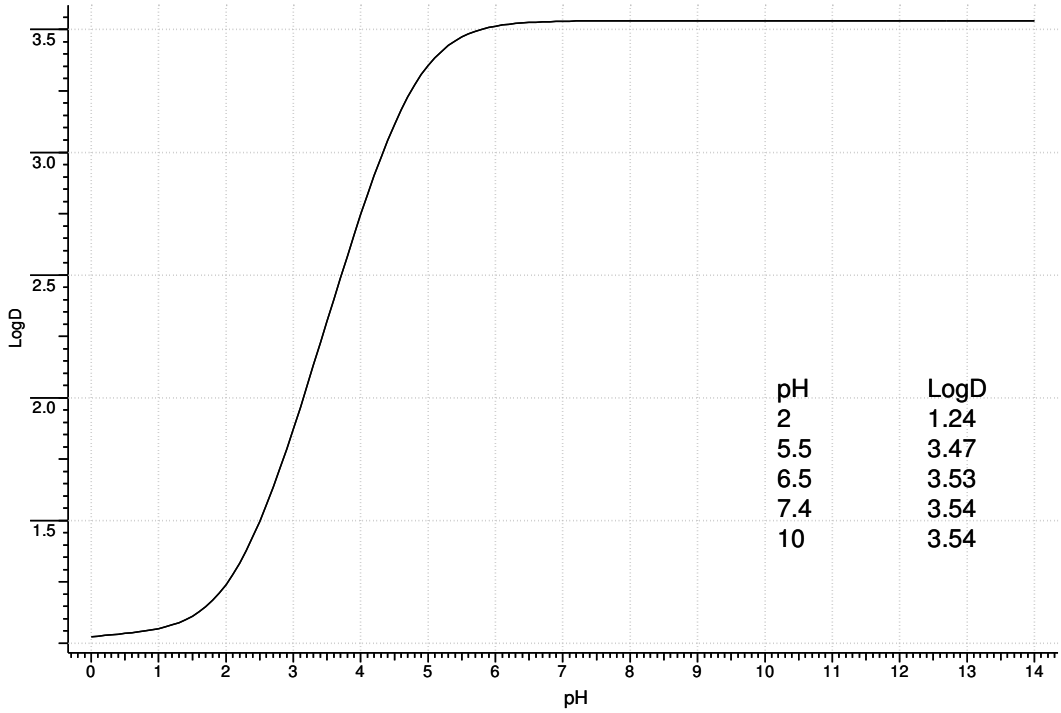


Figure 6-2; Effect of pH on Log D values for SARX

A deuterated stable isotope analogue was available for use prepared by substitution of the ^{1}H hydrogen atoms for ^{2}H on both methyl groups in the dimethoxy benzyl group. This resulted in a heavy labelled compound with a molecular mass of 610.6. The difference of 6 mass units from the unlabelled analyte was sufficient to prevent cross-talk in mass spectrometer response as a result of the isotopic distribution of the two chlorine atoms in the structure. The hexadeuterated analogue was therefore sufficiently discriminative for use as an internal standard for discriminative mass chromatogram quantification.

6.2 Method Optimisation/Validation

Preparation of standard solutions

Stock standard solutions (100 µg/mL) were prepared in DMSO from SARX hydrochloride salt and stored under ambient conditions. The stock standards were further diluted with DMSO to yield standard solutions of 0.01, 0.02, 0.05, 0.10, 0.50, 1.00, 2.00 and 3.00 µg/mL. Internal standard solution of 4-Chloro-3-[(3R)-5-chloro-1-(2,4-bis-trideuteromethyoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl]-N-ethyl-N-(3-pyridinylmethyl)benzamide (0.100 µg/mL) was prepared in DMSO.

Preparation of human plasma analytical samples

Plasma calibration samples were prepared by mixing control human plasma (4.95 mL) with an aliquot (50 μL) of the SARX standard solutions to give concentrations of 1, 2, 5, 10, 50, 100, 200 and 300 ng/mL. Analytical samples were processed immediately or stored frozen at -20 ± 10°C. A second set of validation test standard samples were prepared in control human plasma at 1, 2, 10, 300 ng/mL. Working internal standard sample (10 ng/mL) was prepared by mixing internal standard solution with K_2EDTA derived control human plasma.

Preparation of samples for analysis

Each plasma sample (50 μL) was added to a 96 deep well polypropylene microtitre plate and mixed with 50 μL of working internal standard ([$^2H_6$]-SARX in plasma). The plate was sealed with a pierceable square well polypropylene sealing cap and the plate centrifuged (4000 rpm; ambient temperature) for 10 minutes to enable maximum contact between small volume components. The samples were mixed for 10 minutes on a vortex plate mixer and then centrifuged for a further 10 minutes (4000 rpm; ambient temperature) to remove suspended particulates from the plasma sample and also to ensure that the small volume of plasma was collected at the bottom of the microtitre plate following mixing to ensure aspiration of the complete aliquot volume by the autosampling injector. An aliquot of the plasma (40 μL) was then analysed by Turboflow® LC-MS/MS using the method described below.

Chromatographic procedure

50 μL plasma samples were injected onto the Turboflow (C2; 50 x 1.0 mm i.d., 50 μm) column in pH 3 20 mM ammonium formate at 5 mL/min for 30 seconds allowing plasma proteins and macromolecules to elute to waste while SARX is retained. The analyte was then eluted from the turboflow column by pumping pH 3, 20 mM ammonium formate/acetonitrile (60:40 v/v) through the column at 0.5 mL/min in the reverse direction of loading by means of the valve interface module (Table 6-1). The eluent flow was split using a flow splitter and 250 μL/min diverted to the mass spectrometer.

The chromatographic separation was achieved by diverting the eluent of the Turboflow column through the analytical BetaBasic column (C8; 50 x 1.0 mm, 50 μm) and applying a gradient of 40 to 75% acetonitrile over 2.0 minutes (Table 6-1).

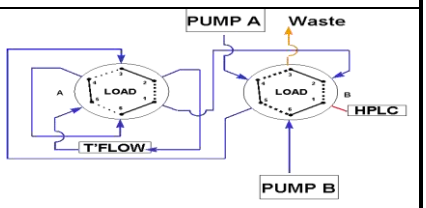
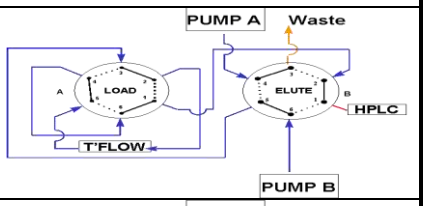
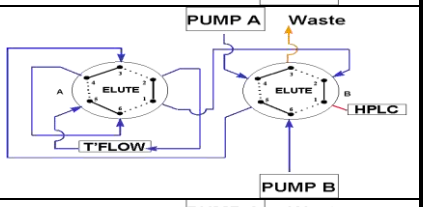
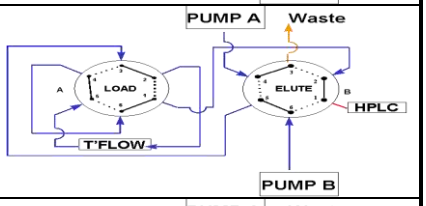
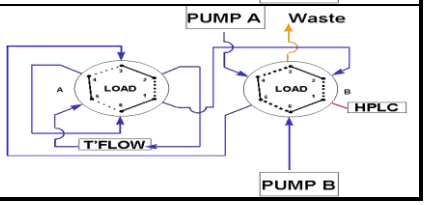
	Loading Mobile Phase Pump (Pump A)			Analytical Mobile Phase Pump (Pump B)				Valve positions and flow path
Time (min)	Flow (mL/ min)	% B	Valve 1*	Flow (mL/ min)	% B		Valve 2*	
0.00	5.0	0	LOAD	0.5	40	Step	LOAD	
0.75	5.0	0	LOAD	0.5	40	Step	ELUTE	
1.00	5.0	0	ELUTE	0.5	40	Step	ELUTE	
1.75	5.0	100	ELUTE	0.5	75	Ramp	ELUTE	
3.75	5.0	100	LOAD	0.5	75	Step	ELUTE	
4.08	5.0	100	LOAD	0.5	75	Step	ELUTE	
4.67	5.0	0	LOAD	0.5	40	Step	LOAD	

Table 6-1; Chromatographic sequence

Mass spectrometry

The mass spectrometer was operated using electrospray ionization under the conditions described in Table 6-2:

Experimental Method Parameters	MS Acquire time		
	Type	Multiple Reaction Monitoring	
	Ion Mode	ESI +ve	
	Channel 1	Precursor m/z	604.1 Da
		Product m/z	151.0 Da
		Span	0
		Dwell (sec)	0.3
		Delay (sec)	0.03
	Channel 2	Precursor m/z	610.1 Da
		Product m/z	157.0 Da
		Span	0
		Dwell (sec)	0.3
		Delay (sec)	0.03
ESI Source Parameters	Source Temperature (ºC)	130	
	Desolvation Temperature (ºC)	375	
	Collision Gas Pressure (MBar)	1×10^{-3}	
	Cone Gas Flow (L/h)	55	
	Desolvation Gas (L/h)	900	
	Cone Voltage (V)	40	
	Capiliary Voltage (kV)	1	
	RF Lens 1 (V)	5	
	Aperture (V)	1	
	RF Lens 2 (V)	0	
Typical Analyser Parameters	LM1 Resolution	14	
	LM2 Resolution	14	
	Ion Energy 1	1	
	Entrance	-1	
	Collision	25	
	Exit	2	
	LM1 Resolution	14	
	LM2 Resolution	14	
	Ion Energy 1	2	
	Multiplier	650	

Table 6-2; Mass spectrometer operating parameters

Results and discussion

An LC-MS/MS mass chromatogram was generated from the selected reaction monitoring trace of 604.1 Da → 151 Da for SARX and 610.1 Da → 157 Da for [$^{2}H_{6}$]-SARX. A representative precursor and product ion scan for 4-chloro-3-[(3r)-5-chloro-1-(2,4-dimethyoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1h-indol-3-yl]-n-ethyl-n-(3-pyridinylmethyl) benzamide is shown in Figure 6-3 together with the proposed fragmentation and the SRM LC-MS/MS chromatogram of the compound and its 6-deuterated internal standard at the lower limit of quantification is shown in Figure 6-4.

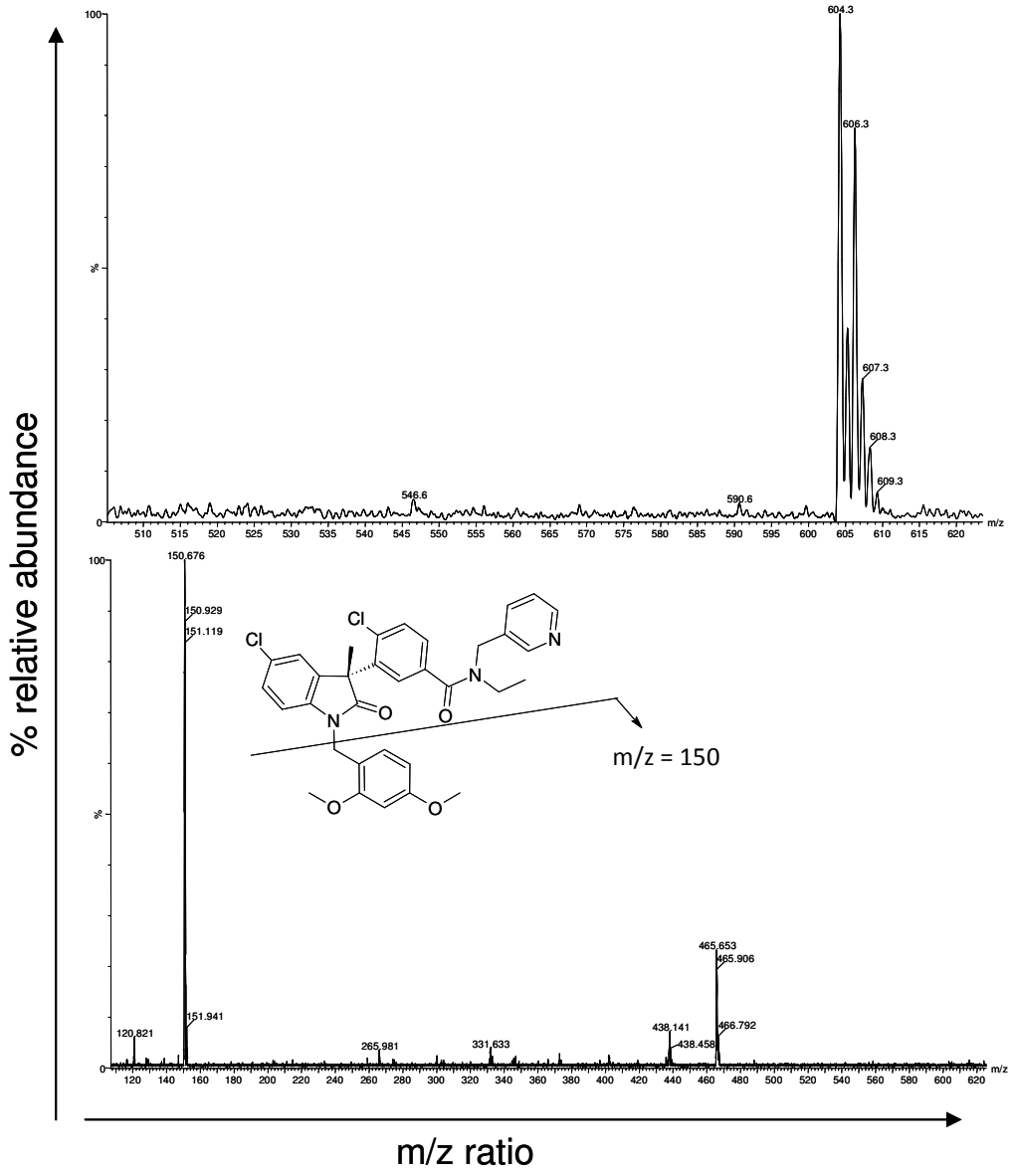


Figure 6-3; Mass spectrum (top) of 4-Chloro-3-[(3R)-5-chloro-1-(2,4-dimethyoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl]-N-ethyl-N-(3-pyridinylmethyl)benzamide and product ion spectrum (bottom) showing proposed fragmentation of the most abundant product ion

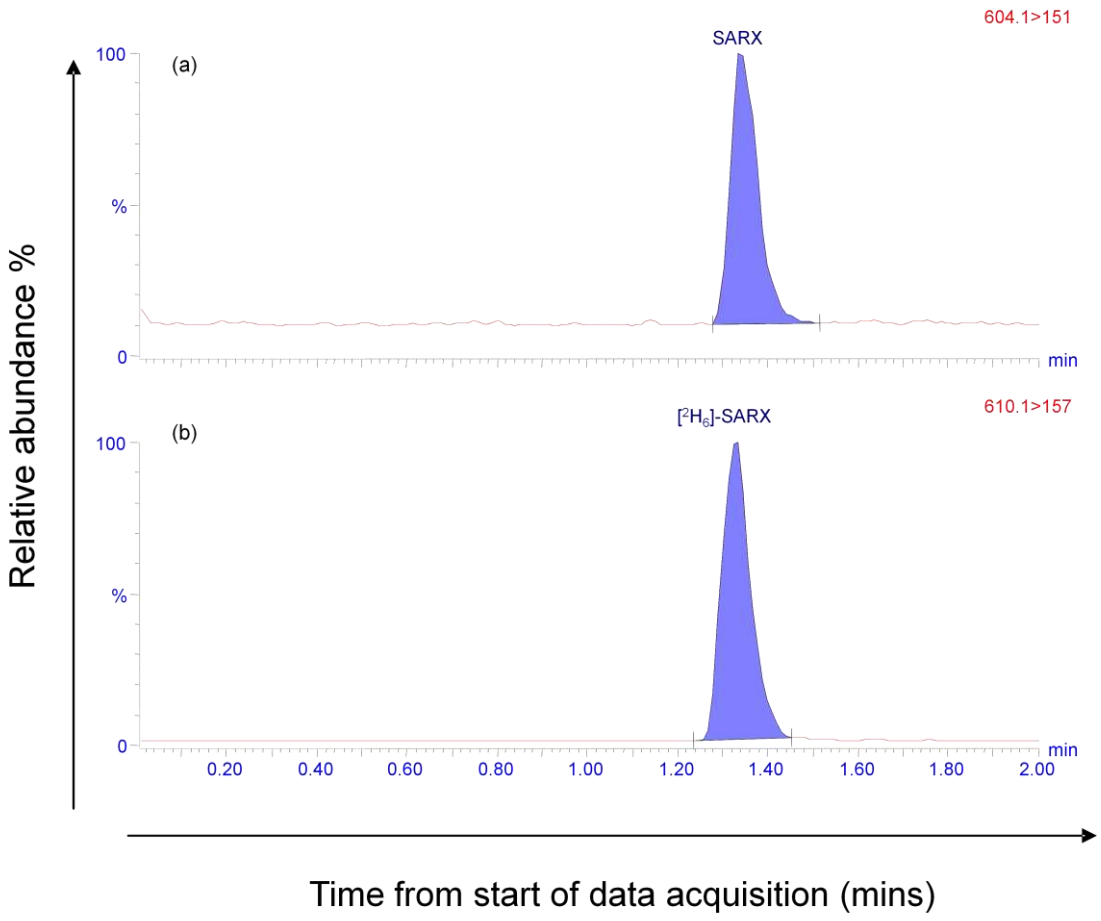


Figure 6-4; Representative product ion chromatogram of control human plasma spiked with (a) SARX (1 ng/mL) and (b) [2H6]-SARX

Calibration linearity, lower limit of quantification and absolute dynamic range.

The absolute sensitivity of the assay was determined as at least 20 pg on column from plasma. This originates from a 40 µL injection of an aliquot of test sample (1 ng/mL) diluted 50% with internal standard. The resulting chromatogram produced a peak for SARX which gave a signal to noise significantly greater than 5-fold when compared to the amplified baseline shown in Figure 6-5. The calibration response was found to be linear between 1 ng/mL and 300 ng/mL in plasma ($y = 0.1086x – 0.02101$; $R^2 = 0.9999$). The inter-run and intra-run precision and bias was assessed at 4 concentrations across the calibration range by preparing 3 replicate groups of control samples and analysing them on 6 separate occasions (6 separate calibration lines). The suitability of the assay for the determination of test samples was determined since each measure of precision and bias were within regulatory limitations of ± 15% (± 20% at lower limit) of expected (Table 6-3).

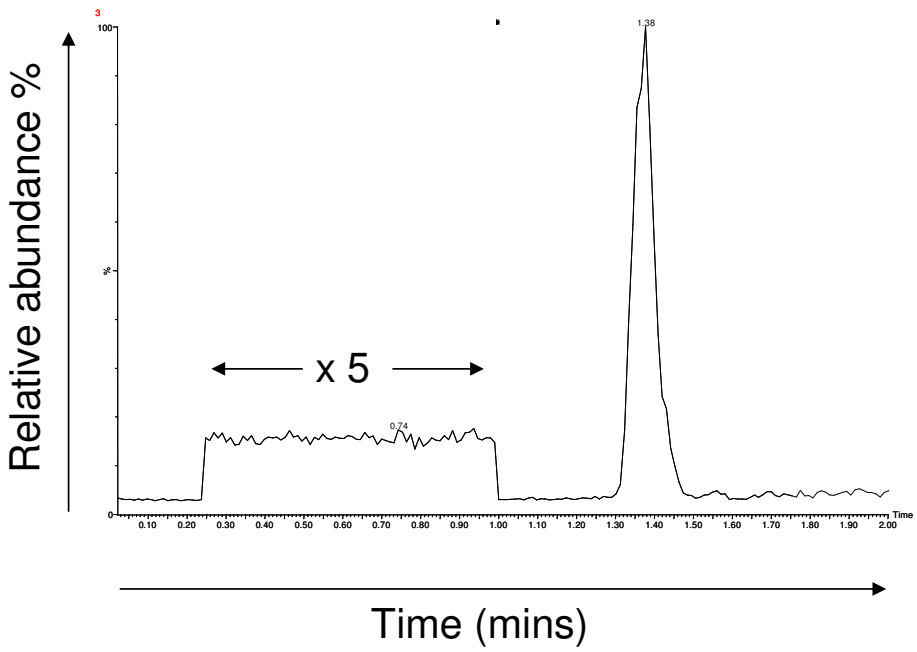


Figure 6-5; Representative SRM chromatogram of control human plasma spiked with SARX (1 ng/mL)

Nominal Concentration (ng/mL)	Plasma Concentration (ng/mL)			
	1.00	**2.00**	**10.0**	**300**
Mean	0.982	1.91	10.3	303
Bias (M%D) **(95% Confidence Interval)**	-1.85 (-5.25, 1.55)	-4.28 (-7.52, -1.03)	2.82 (-3.45, 9.09)	0.944 (-0.216, 2.11)
intra-run Precision (%CV) **(95% Confidence Interval)**	4.25 (3.04, 7.01)	7.10 (5.09, 11.7)	9.00 (6.45, 14.9)	6.67 (4.78, 11.0)
inter-run Precision (%CV) **(95% Confidence Interval)**	2.21 (0.00, 7.69)	0.00 (*, *)	2.60 (0.00, 13.2)	0.00 (*, *)
Total Precision (%CV) **(95% Confidence Interval)**	4.79 (3.75, 9.02)	7.10 (5.09, 11.7)	9.37 (7.38, 16.8)	6.67 (4.78, 11.0)

*, confidence interval not calculated

Table 6-3; Inter- and intra-run precision and bias for the assay in human plasma

The impact of plasma matrix batch was also reviewed by analysing replicate samples in plasma obtained from 3 human volunteers of either sex. Acceptable precision and bias data within ± 15% nominal showed that the assay could be successfully applied without significant bias from subject or sex related variability (Table 6-4).

Matrix Variability	Plasma Concentration (ng/mL)						
	1 ng/mL						
Plasma Lot Number	**Male 1 (n = 6)**	**Male 2 (n = 6)**	**Male 3 (n = 6)**	**Female 1 (n = 6)**	**Female 2 (n = 6)**	**Female 3 (n = 6)**	**All lots (n = 36)**
Mean	1.09	1.09	1.14	1.02	1.01	1.01	1.06
Precision (%CV)	8.27	7.15	7.94	8.21	6.96	8.75	8.70
Bias (M%D)	9.00	9.00	14.0	2.00	1.00	1.00	6.00

Table 6-4; The impact of subject variability and gender on assay precision and bias

Biological stability and solution robustness.

The long term robustness of a pharmaceutical compound in solution is determined by the physicochemical stability of the compound in the dissolution media or solvent, its predisposition to undergo oxidative or reductive reactions which gradually degrade the compound into alternative products. Loss of solution robustness may also be caused by instability of the solvent to chemical degradation or evaporation and gradual precipitation or non specific binding of the compound to storage vessel surfaces. The net result of these changes over time is a (negative) change in compound concentration in solution. Pharmaceutical and exogenous molecules contained in blood plasma may also undergo concentration changes as a result of both chemical and biological factors [193] where even under frozen conditions, metabolic degradation reactions may be enzyme catalysed. Therefore, solution robustness of the compound was evaluated in DMSO solvent used in the preparation of analytical control samples for up to 12 weeks and biological stability was evaluated in the plasma matrix for the same period of time under frozen conditions.

An aliquot of SARX in DMSO was added to control human plasma to result in nominal concentrations of 2.00 and 300 ng/mL which were analysed immediately or stored under frozen condition at -20°C in aliquots for analysis after 2, 4 and 12 weeks. Simultaneously solutions of SARX in DMSO were stored under ambient conditions and after 2, 4 and 12 weeks, an aliquot of each was mixed with control human plasma to result in sample concentrations with nominal concentrations of 2.00 and 300 ng/mL. At each timepoint, plasma samples were analysed by the method described in section 6.2 and quantified by comparison with a freshly prepared set of calibration samples in human plasma. The results presented in Figure 6-6 show that there was no significant chemical instability during the 3 month period since at both concentration levels investigated, negative trend is not observed and the concentration at 3 months is within 15% of nominal concentration and also within 15% of the detected concentration of the sample at time zero. The data demonstrates the absence of significant biological instability since the detected concentrations after 12 weeks are also within 15% of nominal calculated starting concentration. An apparent slightly negative bias at both levels of the plasma calculated concentrations with respect to those calculated from DMSO are attributed to preparation bias in the sample pool prepared for

frozen stability storage, in addition to the freeze/thaw cycle incurred when the frozen plasma sample is removed from the freezer. [79] It is proposed that Cryoprecipitation of plasma proteins on freezing induce a loss of compound mass in addition to reducing volume. Since calculated concentrations remain within the acceptable window of ± 15% and the difference is very small, this factor is not considered to have a significant effect on quantification. Additionally, the effect is further compensated by the use of calibration samples to construct a calibration curve for the extrapolation of sample data ensuring homogeneity of sample handling.

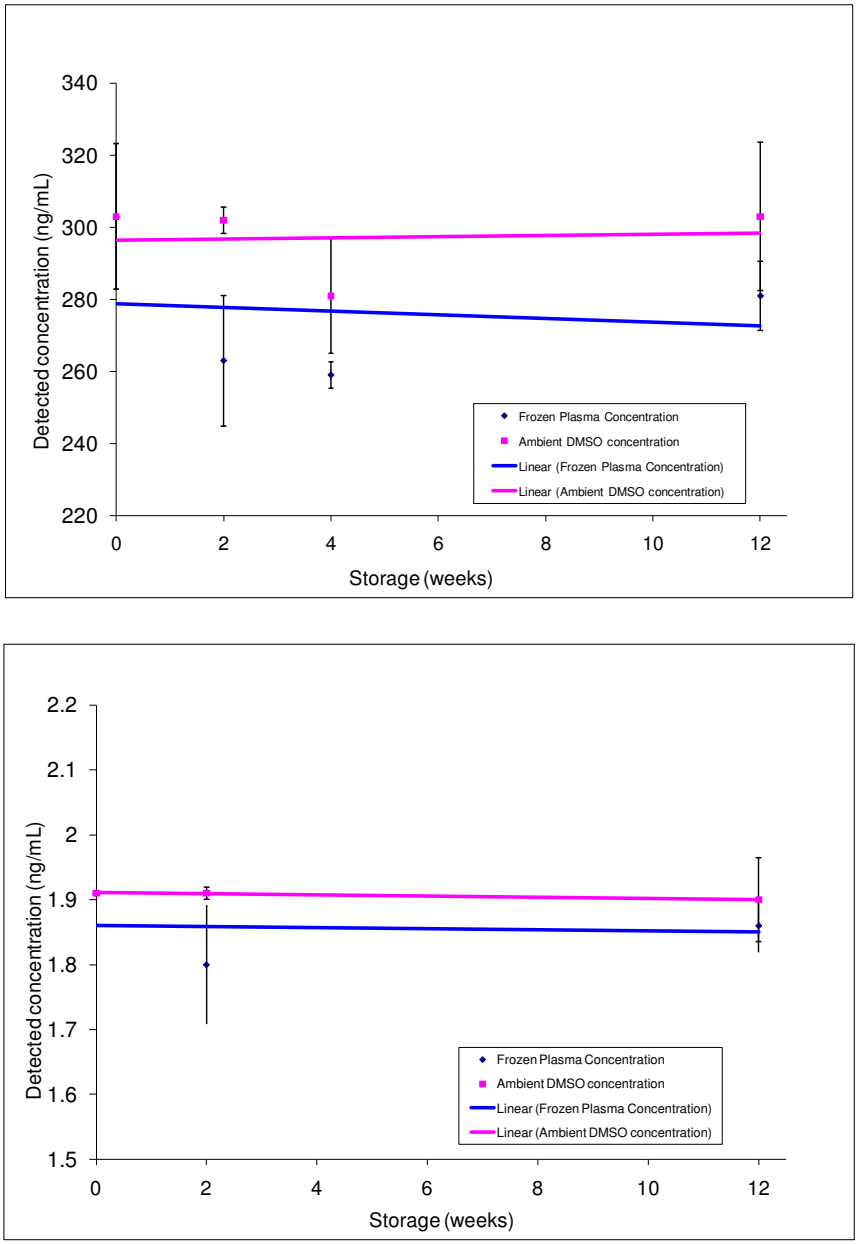


Figure 6-6; Calculated concentrations (± sd) indicating stability of SARX in DMSO under ambient and plasma under frozen conditions at (upper) 300 ng/mL and (lower) 2.00 ng/mL

The absence of degradation or ex-vivo metabolism under the test conditions to greater than 15% deviation from expected concentration indicates that the assay is suitable for use following storage under the test conditions for the period tested (Figure 6-6).

Factors influencing specificity of the assay.

Co-elution of interfering components present in variable concentrations in the test mixture has the potential to disrupt the assay. Chromatographic interference is less significant due to the signal selectivity of precursor to product ion transition although co-elution can cause deterioration of peak shape and ionisation suppression in the mass spectrometer source. [194, 117] The plasma sample aliquot is a complex mixture with a number of variable factors. The origins of compounds present in the mixture may be endogenous biomolecules, xenobiotic compounds such as concomitant medications, or compounds added to the test sample as a result of sample removal, such as blood tube anticoagulant or agents used in the manufacture of the sampling vessels or analytical equipment. The impact of variability in endogenous compounds on the assay was assessed by the analysis of control samples from different individuals by performing matrix variability studies as detailed above. Red blood cell haemolysis to contaminate the plasma sample with cell debris was investigated and found to have no significant effect, as was the effect of collection of blood samples using sodium and lithium heparin and sodium citrate as anticoagulants which may alter the protein distribution in the plasma sample.

The most likely concomitant medications; non steroidal anti-inflammatory drugs, caffeine and nicotine, female pharmaceutical hormones and enzyme inhibition study substrates were all added to plasma at therapeutic concentrations used to prepare test samples of SARX which was analysed to demonstrate specificity. The assay evaluated was validated in accordance with regulatory requirements and used to support preclinical development studies and phase 1 (first into man) clinical trials.

6.3 Surrogate Matrix Evaluation

Control mouse, rat, rabbit, guinea pig and dog plasma were used to prepare test samples in each plasma type to support method validation within each species to comply with regulatory compliant sample analysis conducted in accordance with the OECD principles of Good Laboratory Practice [195] and adhering to the guidelines for bioanalytical method

validation issued by the US FDA [98] such that the validated assay was suitable for use in generating data to support non-clinical safety studies. Samples were analysed at 4 levels, in replicates of 6, across the assay concentration range against a calibration curve prepared separately in plasma from the same species. The results summarised graphically in Figure 6-7 were satisfactory to support the use of the assay for regulatory preclinical studies since the precision and bias were within 15% (20% at lower limit of quantification).

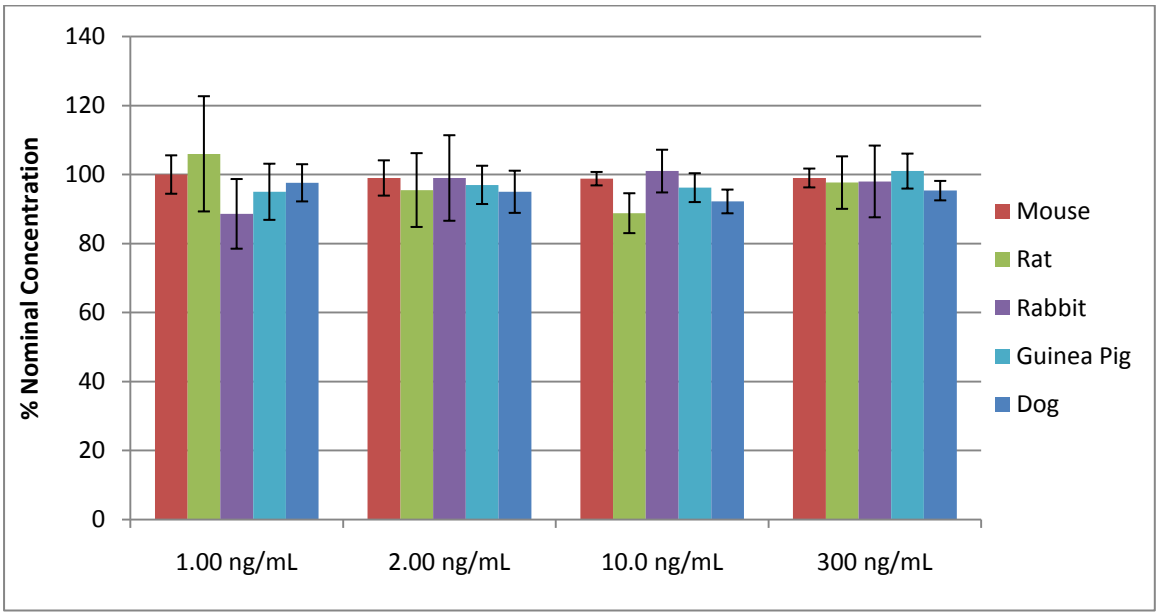


Figure 6-7; Plot of percentage recoveries (± %CV) of samples at concentrations across the assay range (n=6) for test samples in animal plasma analysed against a calibration curve from matrix matched animal plasma.

To evaluate cross species calibration, the experiment was repeated by adding internal standard to each sample from the same batch of human plasma and using a human calibration line in each run to analyse replicates of 6 samples from each species at 4 concentration levels. The dataset obtained is summarised in Figure 6-8.

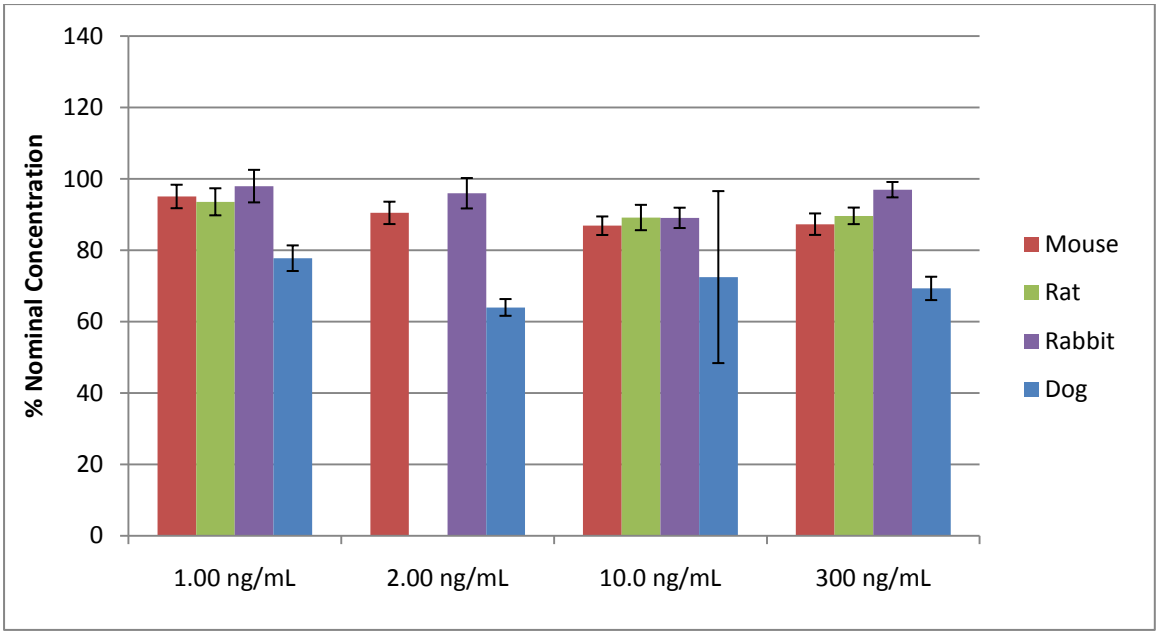


Figure 6-8; Plot of percentage recoveries (± %CV) of samples at concentrations across the assay range (n=6) for test samples in animal plasma analysed against a calibration curve prepared using human control plasma.

While results from mouse, rat and rabbit gave concentrations with acceptable (within 15%/20% at lower limit) precision and bias to support cross-verification, there was significant deviation in detected concentration in dog plasma as indicated by the difference in the lines above while precision data was <15% as expected. A similar observation was made for the analysis of samples in guinea pig plasma. This was due to a suspected species specific matrix effect where endogenous components more abundant in guinea pig and dog plasma co-elute with the analyte and either suppress or induce ionisation efficiency in the mass spectrometer giving rise to non-equivalence of the detector response.

6.4 Ionisation Suppression Investigation

To investigate the effect, the mass spectrometer was configured to operate under post column infusion conditions where a syringe pump was incorporated between the analytical column and the mass spectrometer to deliver a constant infusion of SARX in acetonitrile (100 ng/mL) at 5 µL/min(Figure 6-9). LC-MS chromatograms were acquired at the SRM transition for SARX (604.2 Da → 151.1 Da) so that induction or suppression is observed as broad peaks or troughs, respectively (Figure 6-10).

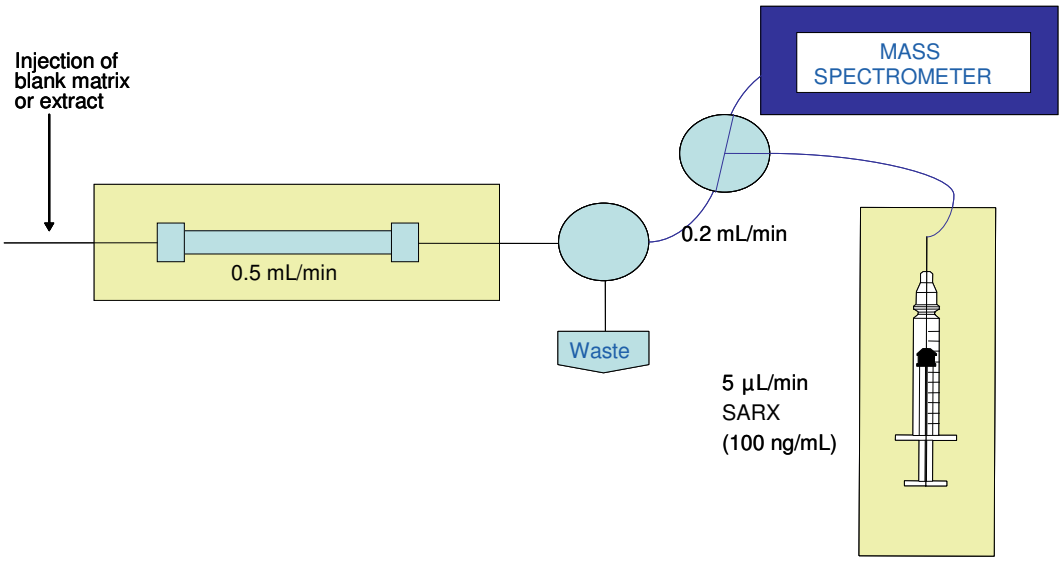


Figure 6-9; Schematic diagram of post column infusion experimental setup

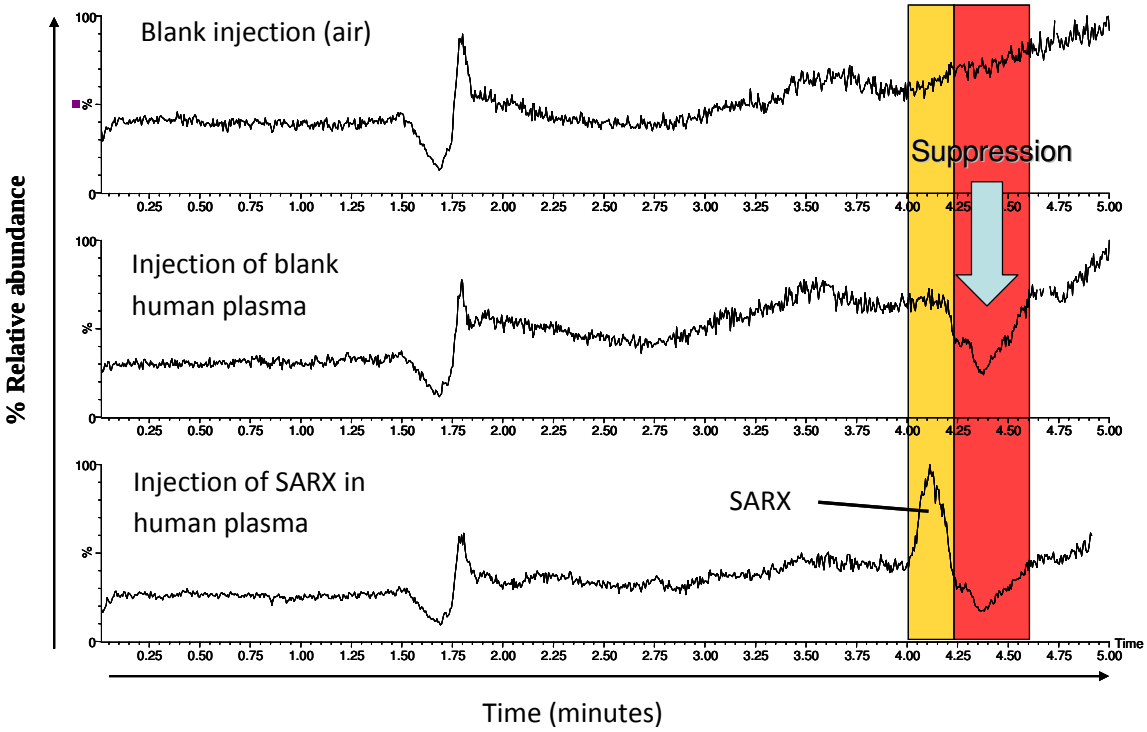


Figure 6-10; Identification of ionisation suppression trough originating from plasma matrix and proximity to analyte retention time.

The post infusion chromatogram of blank plasma in Figure 6-11 shows a suppression zone at approximately 4.25 minutes which is not present in the blank chromatogram. Injection of a sample containing SARX shows that this is close to the retention time of the analyte. In an

attempt to identify the species responsible for the matrix effect, an aliquot of EDTA derived control human plasma was injected onto the system using the post column infusion method to obtain a suppression chromatogram. The infusion pump was removed and the mass spectrometer was set to operate in full scan mode in quadrupole 1 with the collision cell and quadrupole 3 not operating. The resulting chromatogram acquired mass spectra for all species eluting from the chromatography column with the aim of predicting the most abundant species present when zones of suppression are observed.

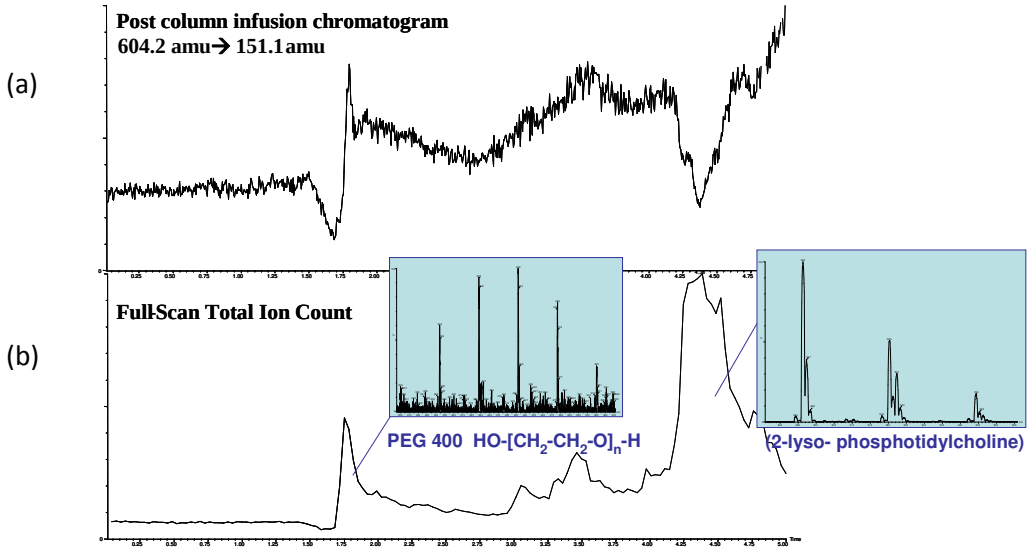


Figure 6-11; Injections of control human plasma (a) post column infusion of SARX monitoring m/z = 604.2 → 151.1 Da (b) full scan mass spectrum.

The experiment identified glycerophosphocholine species present at close to the retention time of the analyte (Figure 6-11). These are endogenous compounds that form a component of cell membranes and are known to give rise to ionisation suppression in bioanalytical assays. [116] Another evident compound class present abundantly although separated from the analyte is polyethylene glycol. A number of compounds containing ethylene glycol polymers may be found in bioanalytical samples residually from the manufacture of sampling equipment but also frequently present in study samples absorbed from intravenous formulations. [112, 196]

Additional full scan chromatograms were obtained for plasma from all test species and found to contain glycerophosphocholine species in a similar profile to those in human

plasma. However, since subject variability has been demonstrated not to have a detrimental effect on quantification, it was postulated that the differential effect on ionisation was due to inter species variations in glycerophosphocholine distribution.

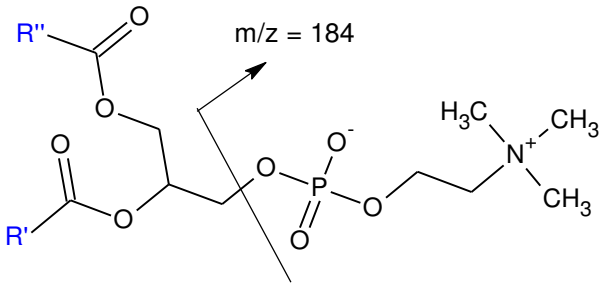


Figure 6-12; General chemistry of glycerophosphocholine species showing common fragmentation

A method to identify glycerophosphocholine species was reported by Little et al. [116] to select species based upon the general fragmentation of the trimethylammonium-ethyl phosphate ion functional group represented in Figure 6-12. The first quadropole of the triple quadropole mass spectrometer was set to full scan, with potential applied to the collision cell and the third quadropole set to m/z 184 Da. The chromatogram was then acquired, generating a precursor ion scan of all species fragmenting to the indicative product of 184 Da. This methodology was applied and a blank plasma sample from each of the test species, together with 3 batches of control human plasma. The precursor ion spectra were obtained at the analyte retention time for each animal species to predict variability in glycerophosphocholine species present. The sum of spectra obtained from 4 to 5 minutes from each chromatogram is presented in Figure 6-13.

These results suggest that the distribution of glycerophosphocholine species is variable between animal species but a more quantitative analysis with selected reaction monitoring for the individual glycerophosphocholines would be necessary to identify the extent of the variation. While it is clear why ionisation suppression occurs to different extents due to the endogenous chemical diversity of different test species, non-equivalence of assay regression was due to variability in the peak area ratio response since these effects effect SARX and its 6-deuterated internal standard to differing degrees. [189, 197] The variability was significant between human, dog and guinea pig and not significant between individuals within the same species.

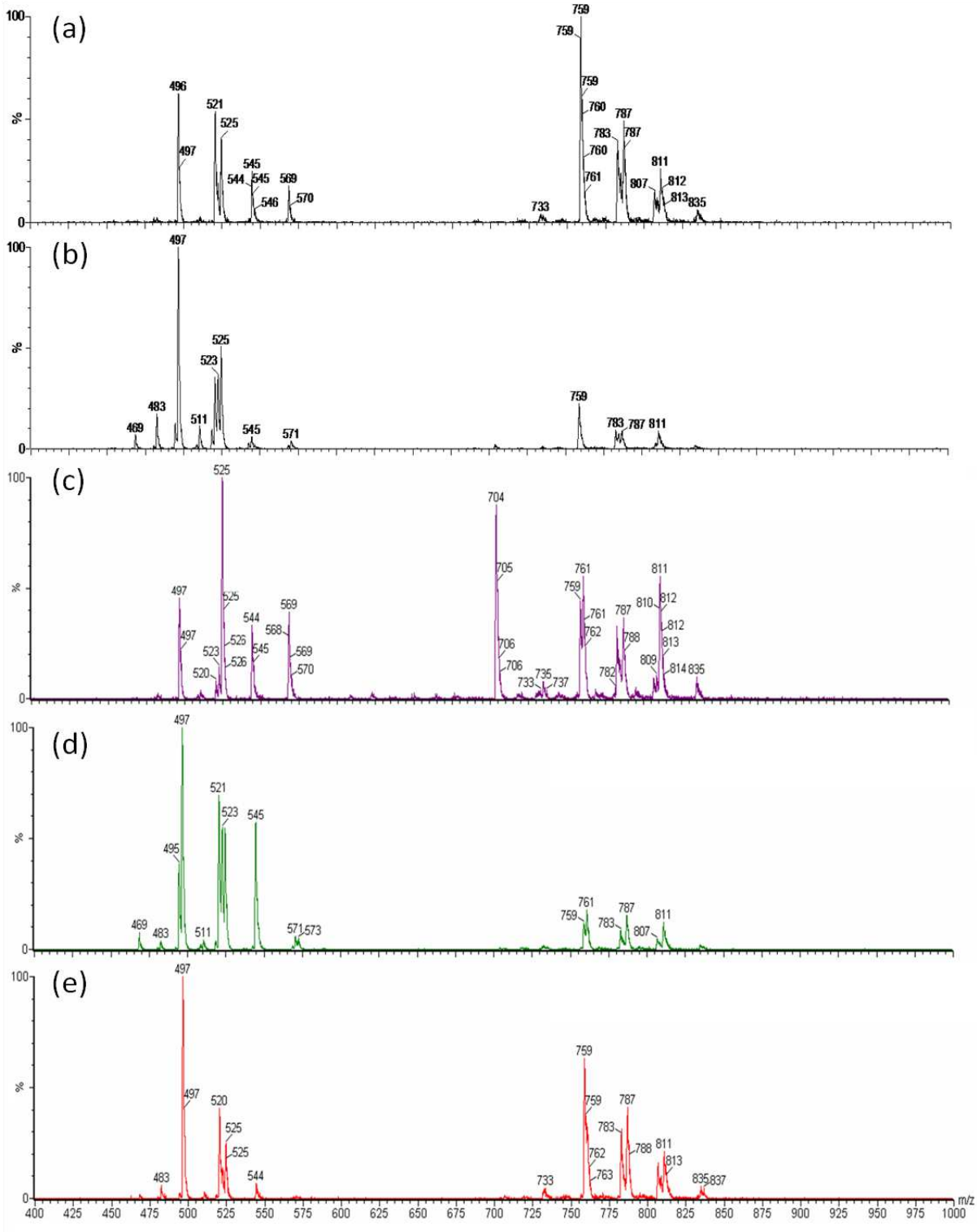


Figure 6-13; Precursor ion spectra of all precursors of product ion mass 184 extracted from 4 to 5 minutes in chromatograms obtained by injection of aliquots of control plasma from animal test species (a) mouse (b) sheep (c) rat (d) dog (e) rabbit

6.5 Differential Ionisation Suppression of Drug and Stable Isotope Labelled Standard

To quantitatively compare the impact of response non-equivalence between animal species without the contribution of variability caused by preparation error variability, a fixed ratio spiking solution was prepared in DMSO containing SARX and [$^2H_6$]-SARX. This method was described by Avery for the characterisation of suppression differences applied to analogue internal standards that were chromatographically resolved from the target analyte. [198] This was added to control plasma from human and animal test species to result in a plasma concentration of 1 and 10 ng/mL for SARX and [$^2H_6$]-SARX respectively. These concentrations represent the lower limit of quantification of the assay where the bias caused by differential ionisation suppression is anticipated to be greatest. A sample from each animal species together with human plasma from 5 different individuals were analysed in triplicate using the SRM method since preparation of a fixed ratio standard eliminated the contribution of preparation error to bias in peak area ratio. Variation in peak area ratio using this test can only be effected by differences in signal response therefore comparative differences between samples are indicative of non equivalent calibration response.

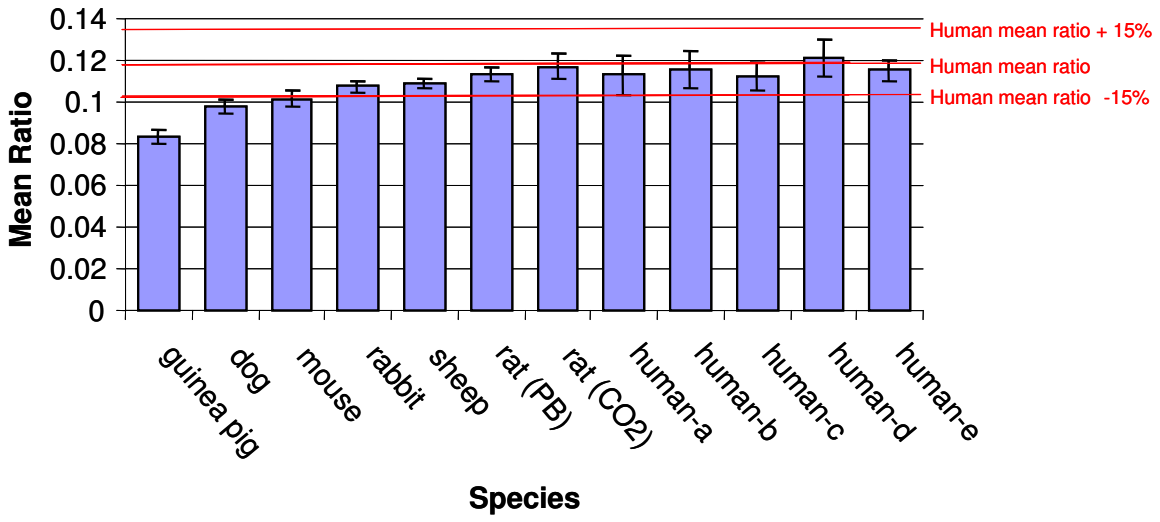


Figure 6-14; The detected peak area ratios (± SD; n=3) of SARX/[2H6]SARX obtained by analysing replicate plasma samples from animal species and 5 different human volunteers prepared using a fixed ratio solution of SARX/[2H6]SARX added to control

The results in Figure 6-14 agree with observations made during cross-calibration experiments that the SARX/[$^2H_6$]-SARX ratio response differs due to species specific matrix effect.

6.6 Proposed Mechanisms for Differential Matrix Effects

The mechanism by which lipid distribution may cause discriminative bias to absolute recovery of an analyte relative to isotopic composition may arise from factors affecting either of chromatographic or ionisation efficiency. The species specific nature of phospholipid composition may consequently infer species specific bias.

6.6.1 Chromatographic effects

Substitution of protons for deuterium atoms are known to affect pKa and therefore chromatographic retention characteristics of the analyte. [202] Transient modification of stationary phase chemistry by association of lipophilic compounds which affect the retentive properties of the phase may consequently affect chromatographic retention of analytes. The extent of this affect may differ between isotopologues with differing pKa values. Since neither difference in chromatographic peak shape nor retention time was observed with respect to the observations shown in Figure 6-14, it is concluded that the influence does not arise from modification of the analytical separation.

The chromatographic mechanism operating on the extraction column may also potentially be affected by stationary phase modification. Here, the differential affect would influence the potential of the stationary phase to overcome association of the analyte with plasma proteins, its absolute retention and subsequent transfer to the analytical column. If the affect was realised, a change in extraction column surface chemistry might bias the recovery ratio of analyte and deuterated standard.

6.6.2 Mass spectrometer ionisation efficiency

Differential ionisation suppression of an analyte and deuterated internal standard have been described, [101, 117, 197, 199, 200] with the explanations *i)* there is some chromatographic resolution between peaks so the two components co elute with slightly different suppressing species [197, 201] or *ii)* different bond lengths of the carbon-hydrogen/carbon-deuterium bond give rise to different pKa values therefore extraction efficiency. [202] In this assay, the chromatographic resolution explanation is unlikely since there is complete overlap between peaks (Figure 6-4), and the differing extraction efficiencies may not completely explain the lack of proportionality giving rise for the relative difference of up to 30% between some matrices with exactly the same proportions of standard added. [198] Co-

eluting phospholipids are present in plasma at concentrations of approximately 300-400 µM for lysophosphotidylcholines, [128, 127] while the reported range of reported Critical Micellar Concentrations (CMCs) of lysophosphatidyl cholines (the point at which surfactant molecules are able to aggregate independently in water to form bilayers or spherical micelles) is between 7 µM to 7 mM. The degree of hydrophobicity of the non polar aliphatic fatty acid chains is inversely proportionate to CMC since micelle formation is favoured, the wide range of physicochemical properties is biochemically important and also explains the broad region of chromatographic interference.

Following plasma extraction and chromatographic retention, it is likely that many coeluting phospholipid species will exceed their CMCs and aggregate into micelles or mixed micelles on elution. Following electrospray ionisation of the column eluent, it has been shown that micelles may enter the gas phase intact, [203] and that glycerophospholipid aggregates may enter the gas phase. [204] In the analytical technique of capillary electrophoresis, [205] the presence of micelles induced by adding surfactants such as sodium dodecyl sulphate to the capillary buffer at greater than CMC have been shown to act as a *pseudo*-stationary phase. This enables the retention and separation of lipophilic analytes or those insufficiently polar to achieve separation in capillary zone electrophoresis (Micellar ElectroKinetic Capillary Chromatography; MEKC). [206] The incorporation of naturally occurring micelles and co-micelles of bile salts has been demonstrated to provide sufficient selectivity to achieve chiral separation. [207] Furthermore, recent work has incorporated phosphotidyl ethanolamine with polyethylene glycol to form the pseudostationary phase for MEKC. [208, 209]

It is proposed that the animal species differences in phospholipid molecular species gives rise to different compositions of glycerophospholipids co eluting with the analyte which form characteristic micelles. Following column elution, an equilibrium exists between partitioning of the analyte and its deuterated analogue in and out of the micelle. The micellar composition is complex and characteristic of animal species and influences the equilibrium of analyte/deuterated analyte in the micelle, resulting in differences in efficiency of ion ejection into the gas phase for mass spectrometry of each analyte ion.

6.7 SARX; Initial Work Conclusions

The assay method described was evaluated and validated sufficiently to support regulated preclinical and clinical pharmacokinetic studies. The potential to cross validate between calibration curves from different species was studied and failure of the process was investigated. While the intra-species calibration was sufficient to support regulated analytical studies, the inter species calibration was not possible in all test species due to the effects of co-eluting endogenous species. A fixed ratio standard spiking protocol was utilised as a comparative predictor of non equivalent ionisation of drug molecules and their stable isotope internal standards in samples of different matrix origins. This is introduced for future application early in method development as a new tool to assess the potential of the assay to cross calibrate between matrices.

Alternative mechanisms of differential extraction chemistry and discriminative pseudo-stationary phase equilibrium biasing relative ionisation efficiency of a compound and its deuterium labelled analogue, each involving co-eluting species specific endogenous glycerophosphocholines are proposed to explain animal species specific differences in matrix effect. Elucidation of the actual mechanism would require the repetition of the fixed ratio test derived from a common control plasma source and conducted in the presence of a continuous infusion of mixtures alternative specific probe choline phospholipids pre-extraction column and post analytical column.

To achieve the objective of interspecies calibration would require re-development of the method to increase extraction selectivity or chromatographic separation. For the assay in question, this would be impractical to avoid impacting on the continuity of ongoing studies.

7 Phospholipid Distribution Investigation

7.1 Introduction

It was proposed that variation in ionisation efficiency between isotopically labelled drug molecules could be effected by differing phospholipid expression between animal species. Initial phospholipid profile screening described in section 6.4 suggested that expressed phospholipid species might differ between animal species. Factors which may affect the distribution of phospholipids are complex and contradictory. Phosphotidyl choline species in plasma are endogenous, being synthesised in the body while dietary phospholipids are metabolised by pancreatic phospholipase enzymes. The compounds have been identified as effective biomarkers of disease, [126, 128] being expressed in response to biochemical stimulus, and contributing to biochemical pathways. However, nutritional biomarker studies have also found that the proportion of unsaturated phosphotidyl choline and other phospholipid species correlate to the intake of dietary polyunsaturated fatty acids, [210-212] most abundantly α-linolenic acid (Figure 7-1) so while phospholipids do not enter the blood stream directly from the diet, dietary influences may also contribute to this variability in expression.

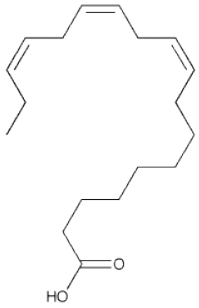


Figure 7-1; α-linolenic acid (octadeca -9, 12, 15-trienoic acid)

The dietary sensitivity of some phosphotidylcholines would suggest observed variability may be attributed to diet while validation of other molecular species as biomarkers of disease state suggests independence from dietary effects. Since membranes can contain a thousand distinct molecular species of phospholipid [213] with diverse structural or biochemical functions, such apparently contradictory behaviour within a class may be understandable.

7.2 Distribution between Animal Species

To characterise the distribution of phospholipids between animal species using an extraction method appropriate to the drug bioanalytical assays under investigation, an HPLC-ESI-MS/MS method using Turboflow extraction (described later in section 8.5) was applied monitoring all precursors of m/z = 184 Da. Lithium heparinised plasma from individual animals across a variety of species obtained as pre-dose (ie, not drug exposed) samples from study animals following completion of study analysis and scheduled for disposal were analysed. Overlaid phospholipid profiles on normalised scales from individual animals of dog, macaque and rat (12, 4 and 18 individual animals, respectively) are presented in Figure 7-2 against an equivalently scaled x axis of time in minutes.

Comparison of these overlaid mass chromatograms support the observation of variability in distribution between species. Since mass chromatograms have been presented on a normalised scale, the largest peak originating from the most abundantly expressed phospholipid is scaled to 100%. Other peaks are plotted rationally which means that the proportionality of the expression of phospholipid peaks within a species group may be compared between species. Peaks evident at 2.25 and 3.75 minutes are expressed proportionately strongly in samples from dog plasma and less in the other species, while in rat plasma, a peak with a retention time of 2.75 minutes is most predominant and in macaque, there is a large peak at 2.8 minutes. Total extracted spectra from 120 to 240 seconds of individual representative animals also support this as represented in Figure 7-3. In each spectrum, the major component is m/z = 497 Da, while in dog plasma, an ion is evident with almost equal intensity at m/z = 525 Da. In rat and macaque plasma, the ion with second greatest intensity is at m/z = 521 Da although in rat plasma this ion is approximately 70% while in macaque plasma it is less than 50%. A peak with m/z = 544 Da is present in each spectrum but with variable intensity while later in the spectra, a peak with m/z = 759 Da is present in dog and rat but close to the limit of detection in macaque.

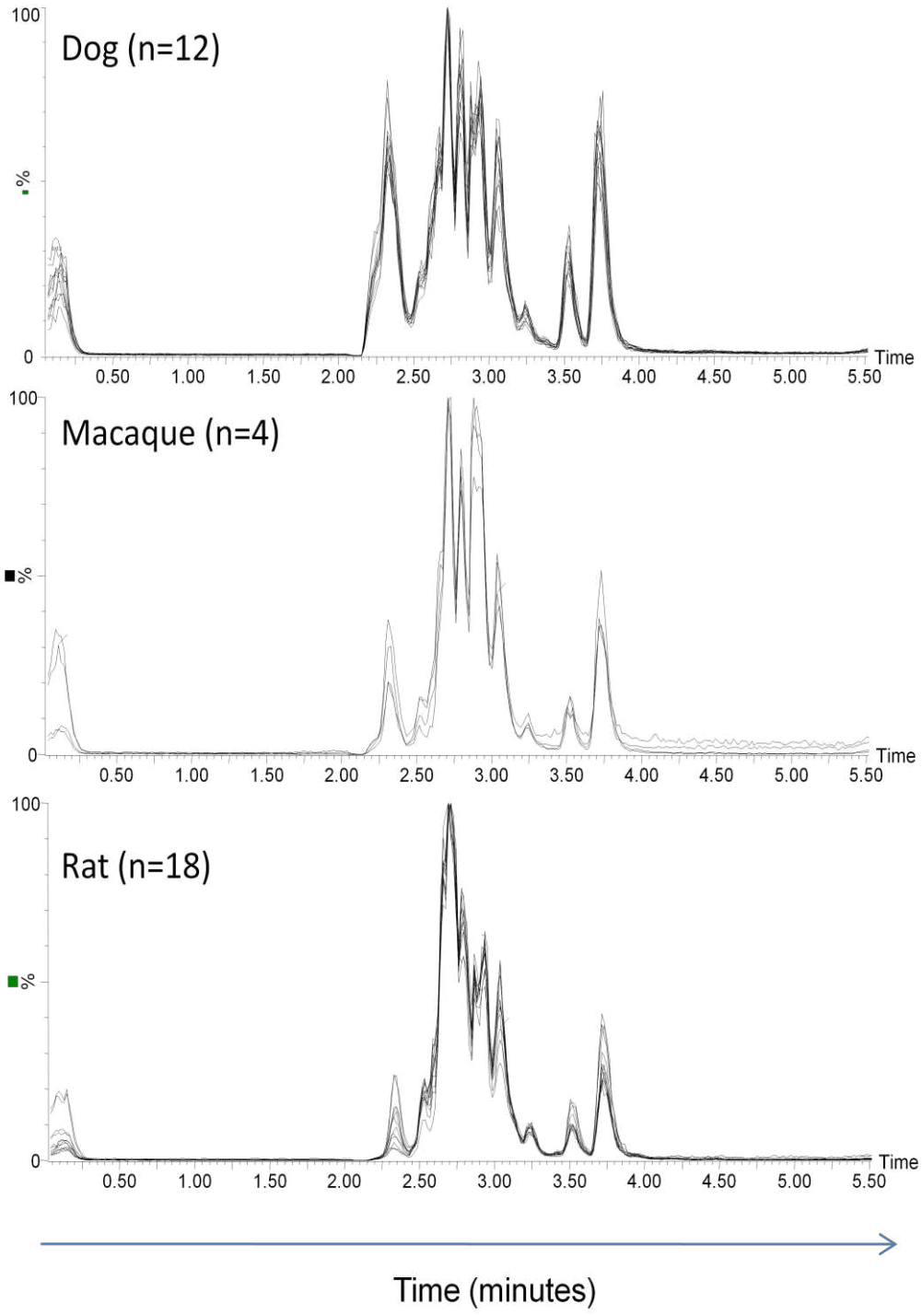


Figure 7-2; Phospholipid profile (total ion count precursors of m/z 184 Da) of 3 different species of overlaid individual animals

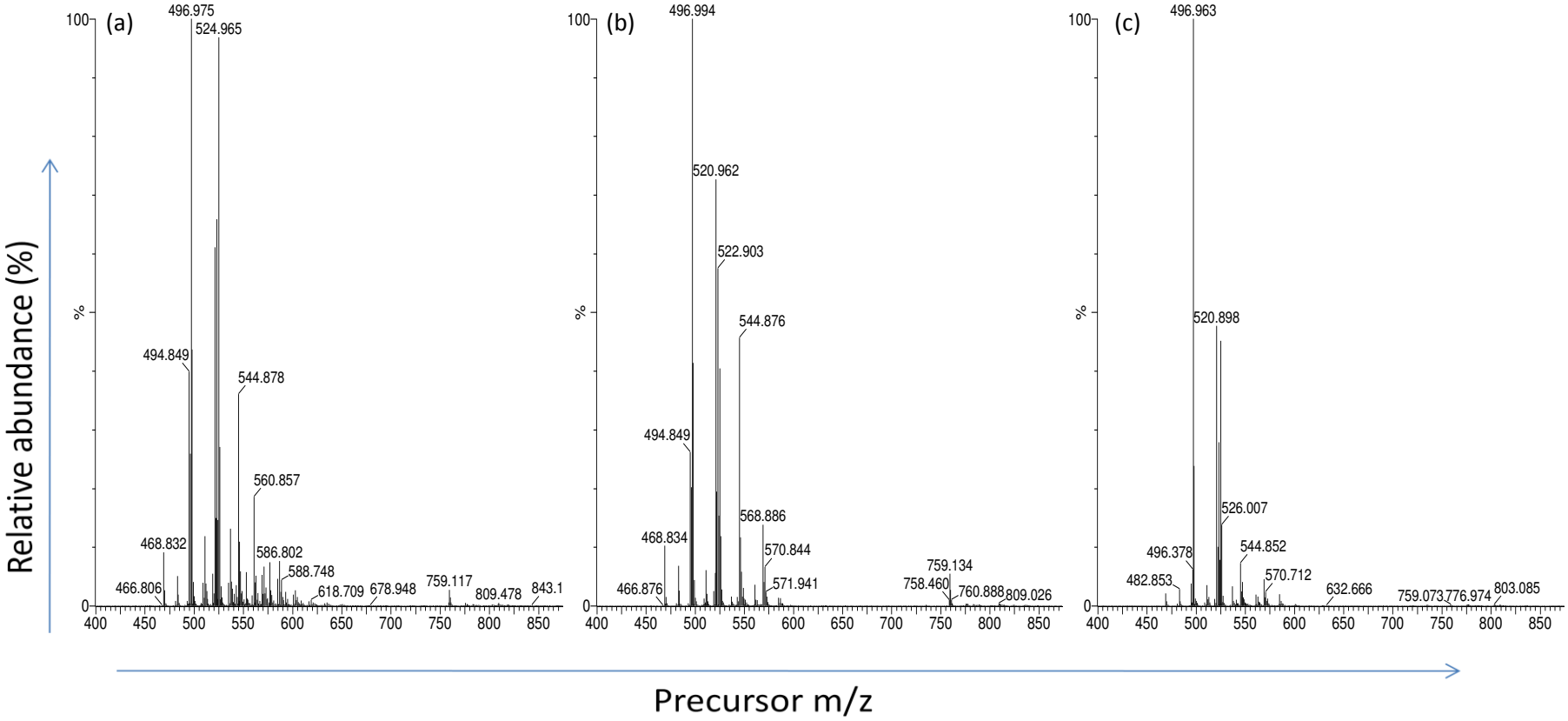


Figure 7-3; Summed ions (precursors of m/z = 184 Da) from 120 to 240 seconds from a) dog b) rat c) macaque

The total ion count mass chromatograms of these samples were used to populate m/z, and time versus ion intensity tables which was imported in to SIMCA P+ software (Umetrics, Umea, Sweden) and used to achieve multidimensional separation by multivariate statistical analysis. The analysis is used to summarise a complex dataset with multiple variables by simplifying and summarising the key data from background noise and recognising differences between variables by mathematical projection. The techniques used were principal component analysis (PCA), where the large number of possibly correlated variables described as eigenvectors in a data matrix is reduced to a small number of uncorrelated variables defined as the principal components of the dataset. This is done by representing each sample together in a mathematically calculated multidimensional space (containing variables of retention time, mass and peak intensity) then calculating a new coordinate axis through which the data represented most variability in the model. This coordinate axis is defined as the first principal component which describes the maximum variability in the dataset, a second axis orthogonal to the first is defined as the second principal component and representing the largest proportion of the remaining variability. Multidimensional data can then be transformed and plotted on a 2 dimensional axis showing the maximum variability in the dataset and used to achieve pattern recognition to gain an overview of the variability features of the dataset. In this case, the patterns may reveal differences between the phospholipid chemical species used to compile the eigenvector for each sample in multidimensional space. Since many phospholipids are present in the sample which may not differ between species, these will not contribute to the calculation of the axes of the first principal components. PCA analysis of the dataset is presented in Figure 7-4.

The data shows separation between groups with one apparent outlier from both the macaque and rat group and while the range of each group on the graph was large, the separation between groups was clear; indicating discernable differences between endogenous phospholipids. Representation by multivariate analysis is more able to elucidate differences between data than visual comparison of spectra as shown in Figure 7-2 since non normalised peak intensities contribute to the differentiation as do minor components and those which may not be chromatographically resolved.

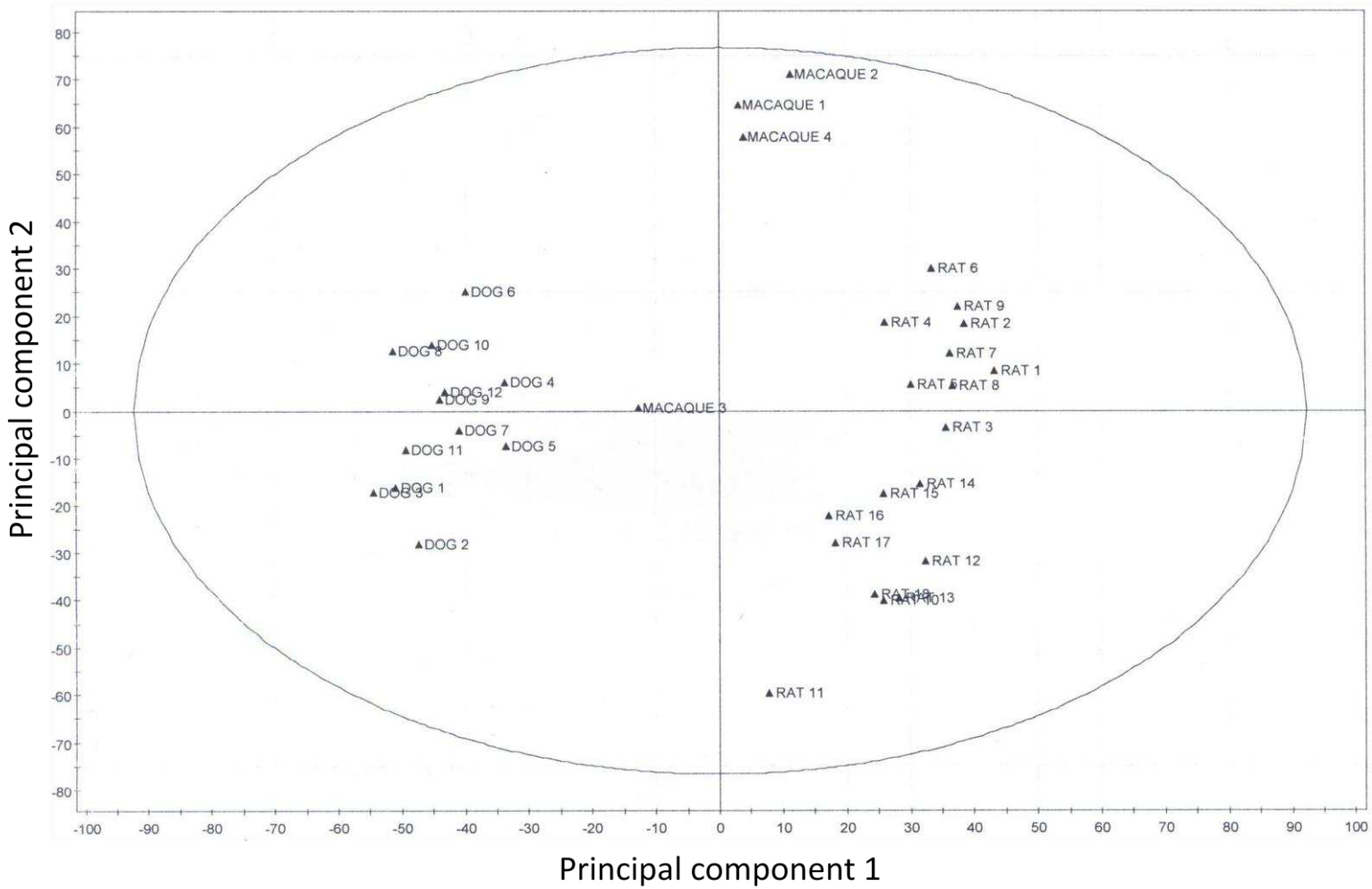


Figure 7-4; Separation of species classes by principal component analysis of phospholipid profile

The second method used is partial least squares regression (PLS), interchangeably called projections to latent structures used as discriminative analysis (PLS-DA). It establishes principal components in the same way as PCA but projects the variables by linear regression into a new space where more of the variability of the dataset are represented (latent variables), therefore separations may be more discriminative and are used to construct predictive models that can be used to classify unknown samples. PLS-DA analysis of the dataset in Figure 7-5 demonstrates greater separation between each group with fewer outliers.

To further test the principal that differences occur between species specific phospholipid profiles, the method was further modified to extend and flatten the analytical gradient allowing greater time separation between molecular species and to achieve elution of more retentive less polar lipids. To reduce the potential for accumulation of persistent phospholipid species and elution in later injections [107] an additional wash solvent cycle of propan-2-ol was incorporated on the quaternary loading pump, which necessitated a reduced flow rate to compensate for the higher back pressure of the more viscous solvent composition while the possibility of biasing the model by any accumulation was reduced by randomisation of samples. A summary of the method is contained in Table 7-1.

To investigate the predictive nature of the separation observed, further lithium heparinised plasma samples from monkey, dog, guinea pig and rat individual animals or separate pools were analysed by this method together with human lithium heparinised plasma samples from healthy volunteers from 2 different ethnic groups (African American and Caucasian origin). A notable observation in the analysis is the low recovery detected from all guinea pig samples (Figure 7-6).

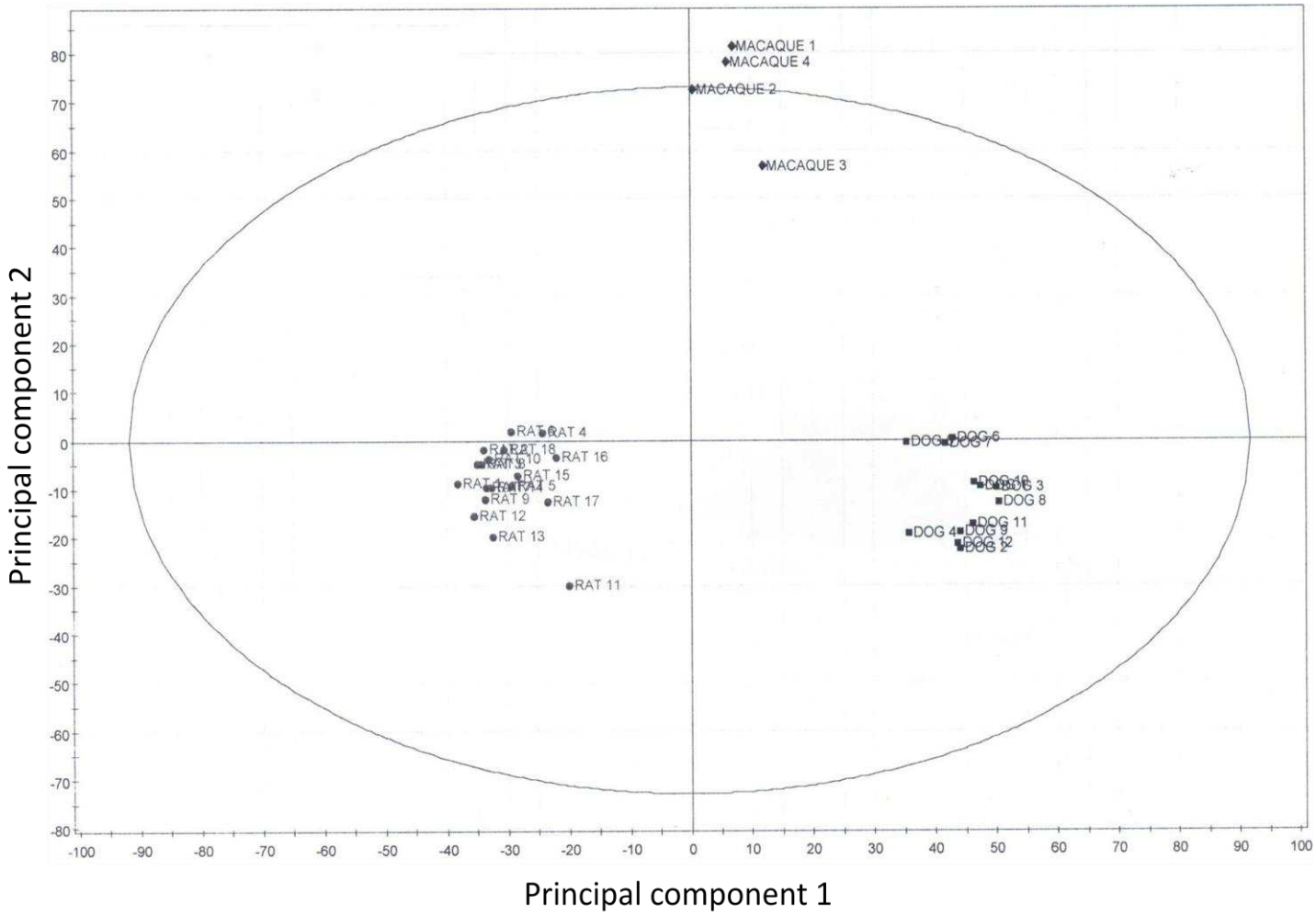


Figure 7-5; Separation of species classes by partial least squares regression discriminative analysis of phospholipid profile

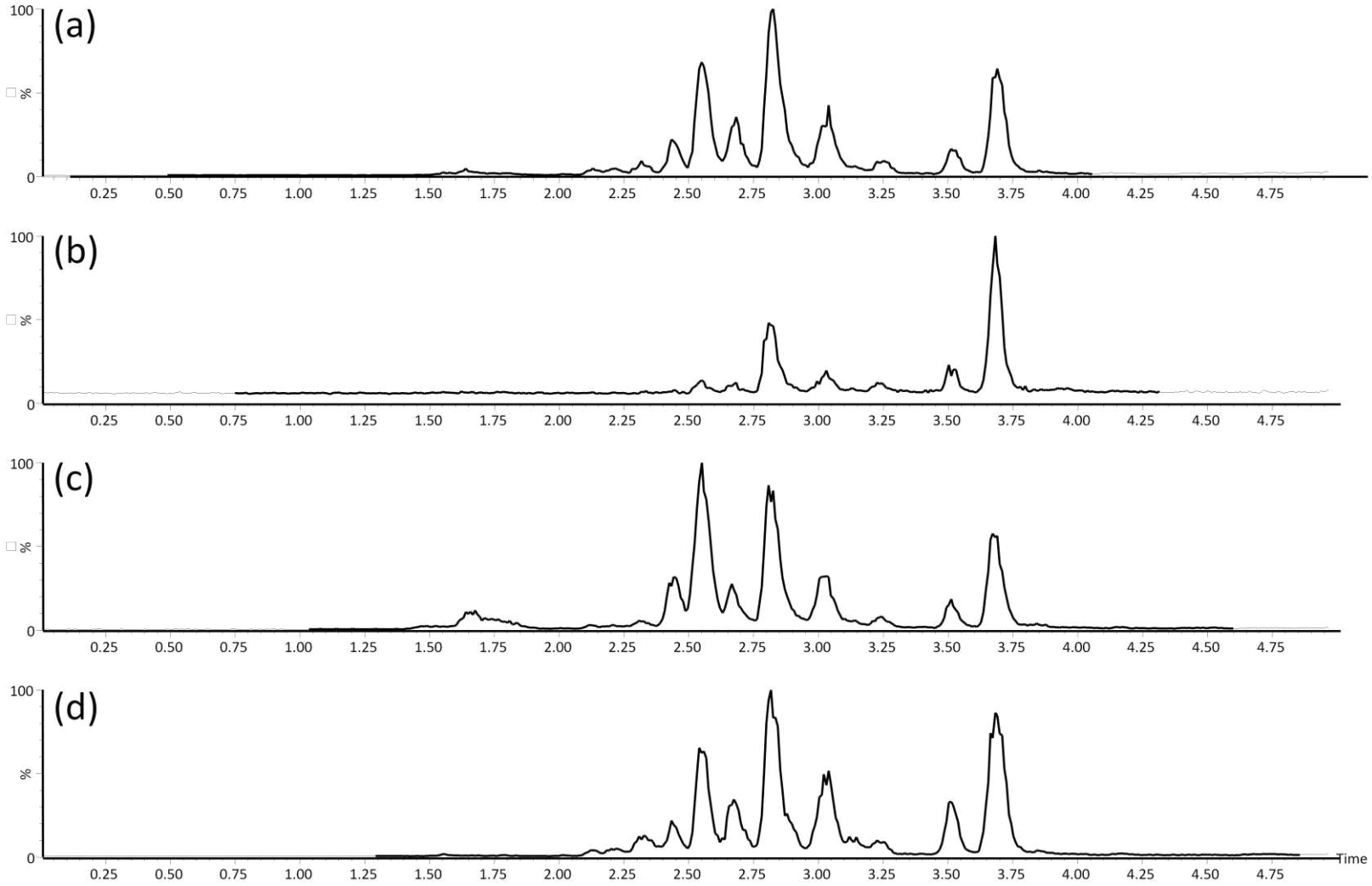


Figure 7-6; Representative Total Ion Count (TIC) mass chromatograms from zero to five minutes of precursors of m/z = 184 Da showing (a) monkey, (b) guinea pig, (c) rat and (d) dog samples

This is consistent with previous research which found that while the most abundant mammalian phospholipids were phosphotidyl choline species, rodents and particularly guinea pigs express greater proportions of phosphotidyl ethanolamine. [214]

Modelling the data was automatically performed with SIMCA P+ software, using partial least squares regression (PLS) analysis and the key PLS components were extracted to identify latent variables represented by the 9 components in Figure 7-7. Here, the results of internal cross validation of the predictive model using each component are used to derive cumulative Q2 values (blue bars) which are an estimate of the predictive separating power of the component selected in the model. Cumulative R2Y values (green bars) which are plotted on the same scale are a measure of the degree of expression of the component within the dataset. [215] The contributions of individual datapoints to the model are represented in the loadings plot shown in Figure 7-8; describing the degree of correlation of the PLS component (mass transition/ chromatographic retention time) with the original variable (species) where positive values of towards (+1) indicate a positive response to the separation and negative values towards (-1) indicate no contribution. The 'VIP list' as defined by SIMCA software containing the most important variables (precursor ion mass/chromatographic retention time) is presented in Table 7-2 (top 50 values). The values are calculated by correlation with X (the other terms) and Y (the projection) and assigned a confidence interval using a jacknife method (data is resampled to calculate the statistic using subsets where individual observations are sequentially excluded, reducing bias).

Representation of this dataset on a 2 dimensional eliptical plot (component 1 plotted against component 2) shows separate grouping of rat, dog and guinea pig samples, while other species show significant overlap.

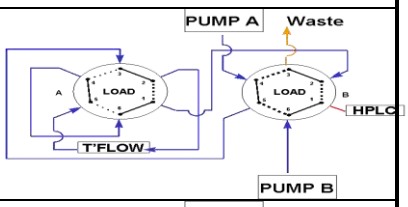
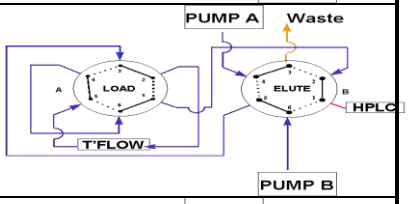
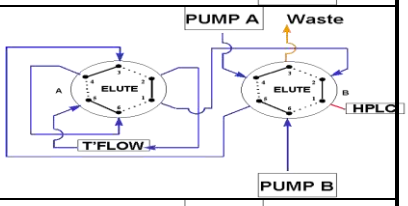
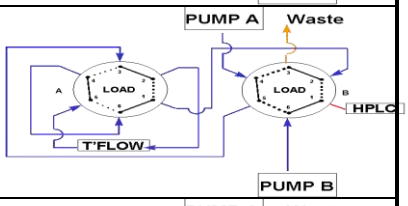
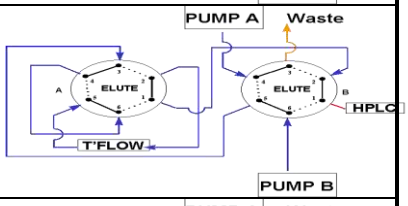
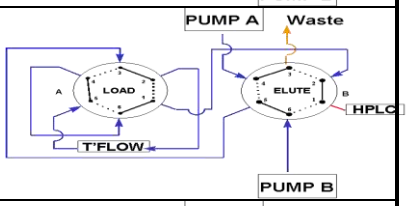
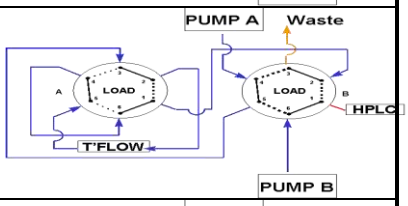
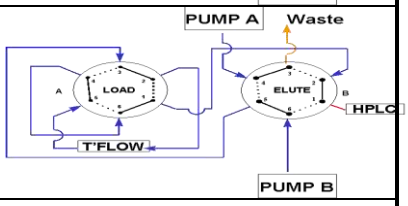
	Loading Mobile Phase Pump (Pump A)				Analytical Mobile Phase Pump (Pump B)				Valve positions and flow path
Time (sec)	Flow (mL/min)	% B	% C	Valve 1*	Flow (mL/min)	% B		Valve 2*	
0	1.25	0	0	LOAD	0.75	55	Step	LOAD	
45	1.25	0	0	ELUTE	0.75	55	Step	LOAD	
60	1.25	0	0	ELUTE	0.75	55	Step	ELUTE	
75	1.25	50	0	ELUTE	0.75	95	Ramp	ELUTE	
375	1.25	0	50	ELUTE	0.75	95	Step	ELUTE	
735	1.25	0	50	LOAD	0.75	95	Step	LOAD	
750	1.00	0	100	ELUTE	0.75	95	Ramp	ELUTE	
765	1.00	0	100	ELUTE	0.75	95	Step	LOAD	
780	1.00	0	100	LOAD	0.75	95	Step	LOAD	
795	1.00	0	100	ELUTE	0.75	55	Step	LOAD	
810	1.00	0	100	LOAD	0.75	55	Step	LOAD	
840	1.25	0	0	LOAD	0.75	55	Step	LOAD	

Table 7-1; Total phospholipid separation chromatographic sequence

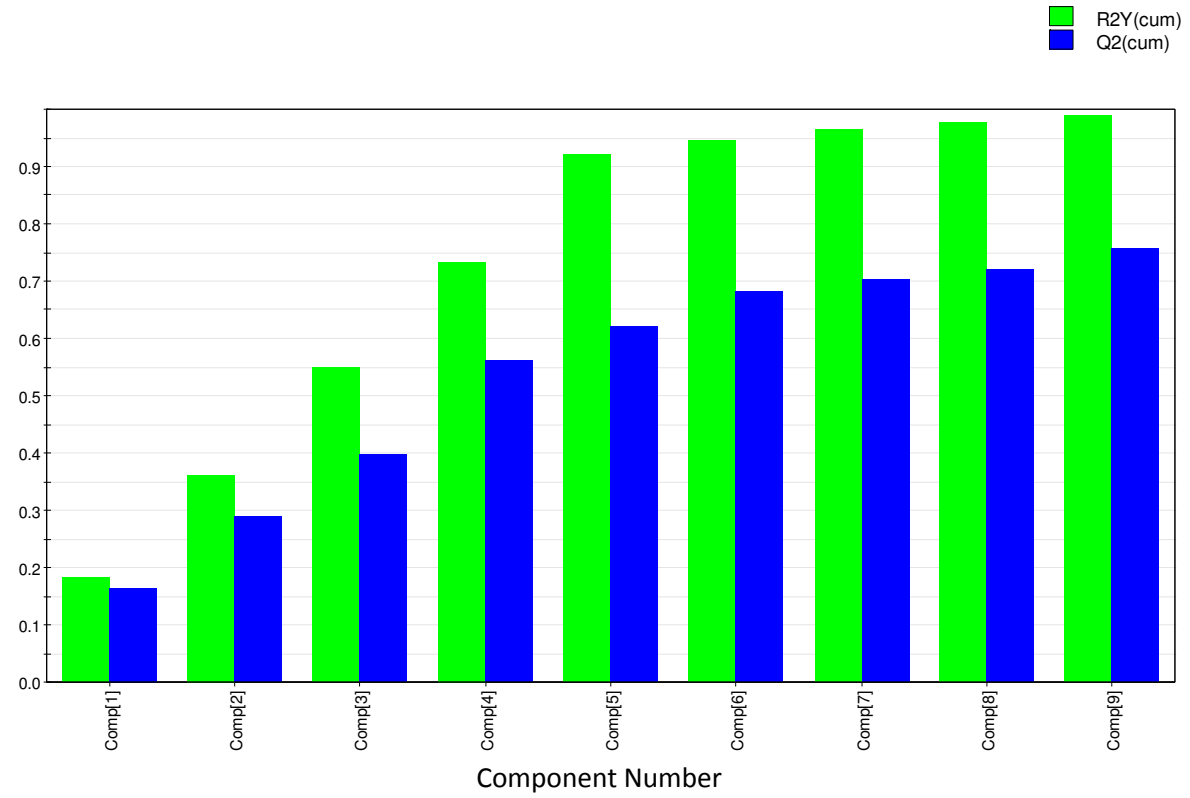


Figure 7-7; Multiple species phospholipids separation; selection of partial least squares component

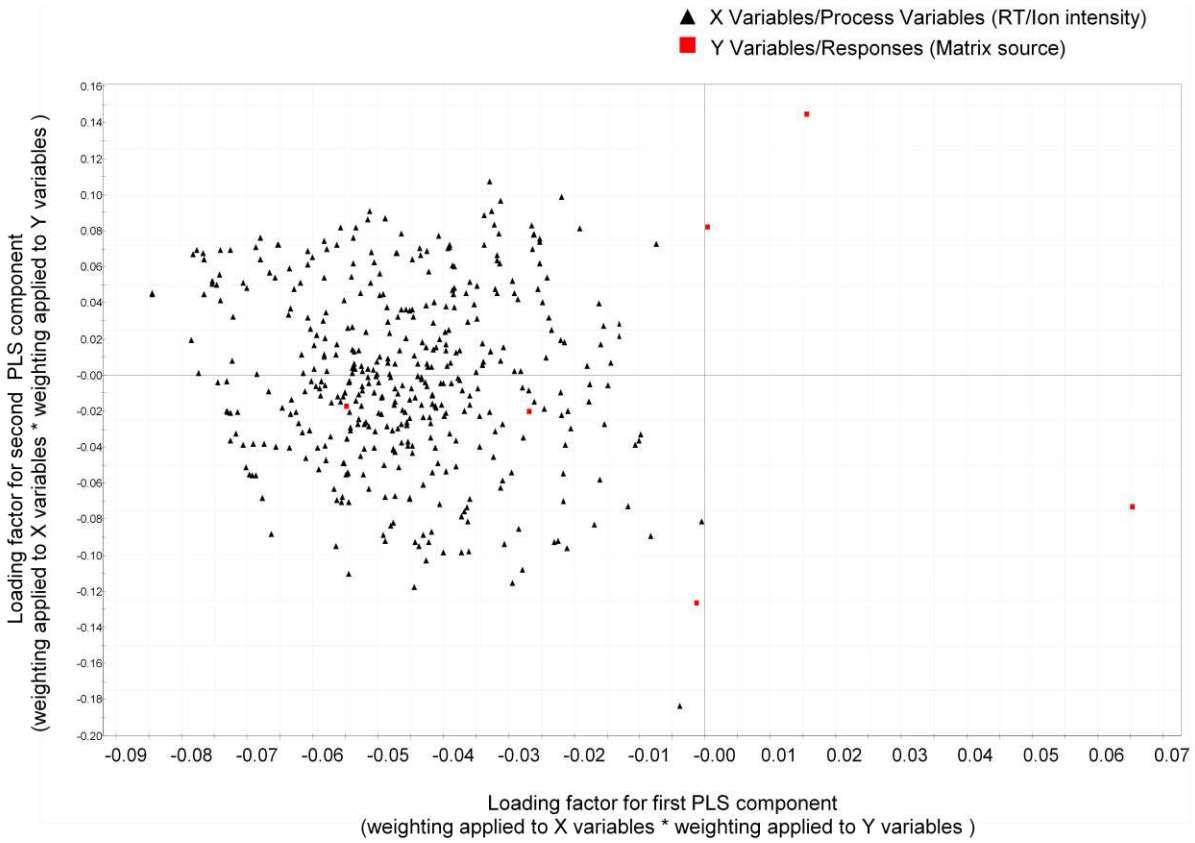


Figure 7-8; Multiple species phospholipids separation; contribution of individual data points to the separation model (loadings plot)

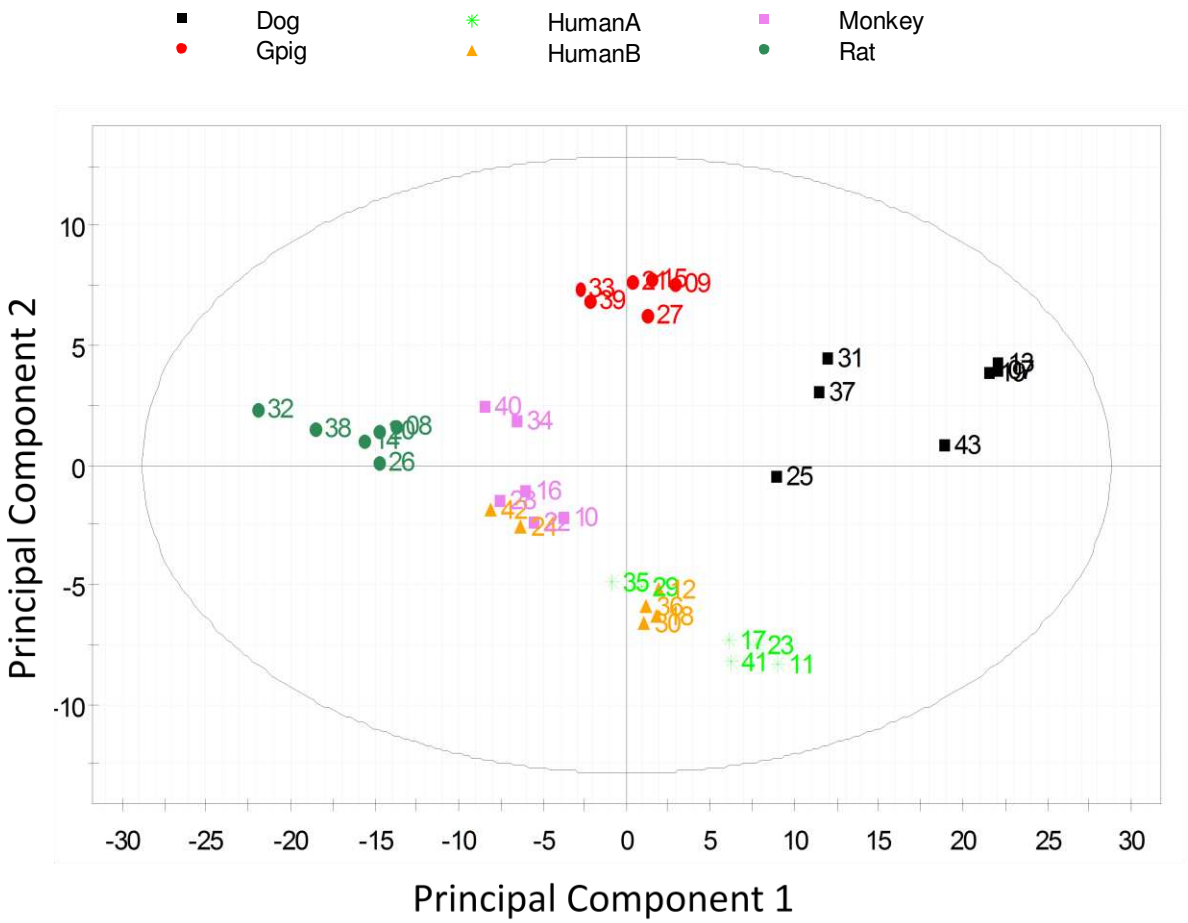


Figure 7-9; 2-dimensional elliptical plot of components 1 and 2 showing all species investigated

Better visualisation of separation of each group can be achieved in 3 dimensions by incorporating a third axis of an alternative PLS component as demonstrated in Figure 7-10 to Figure 7-14. It can be seen that within 3 dimensions, by manipulating the variable plotted on the z axis and rotating the plot, separation may be visually achieved for each component or for several distinct components from the dataset. Manipulation of the first, second and third PLS component can be used to achieve separation of rat, dog and guinea pig from other groups (Figure 7-10 to Figure 7-12) while manipulation of the first and second component with the fourth enable separation of macaque monkey group from the rest (Figure 7-13) and separation of the first and second with the fifth component (Figure 7-14) separate the human groups from animal groups.

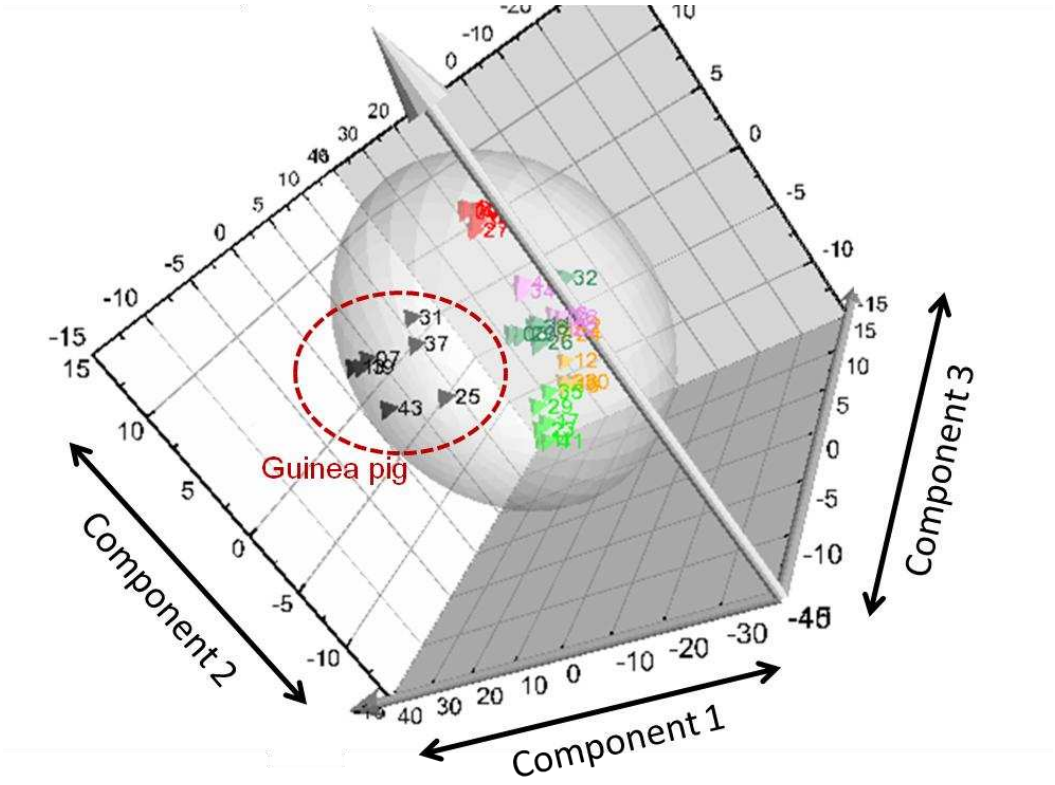


Figure 7-10; 3-dimensional elliptical plot of components 1, 2 and 3 showing separation of the cluster of guinea pig samples

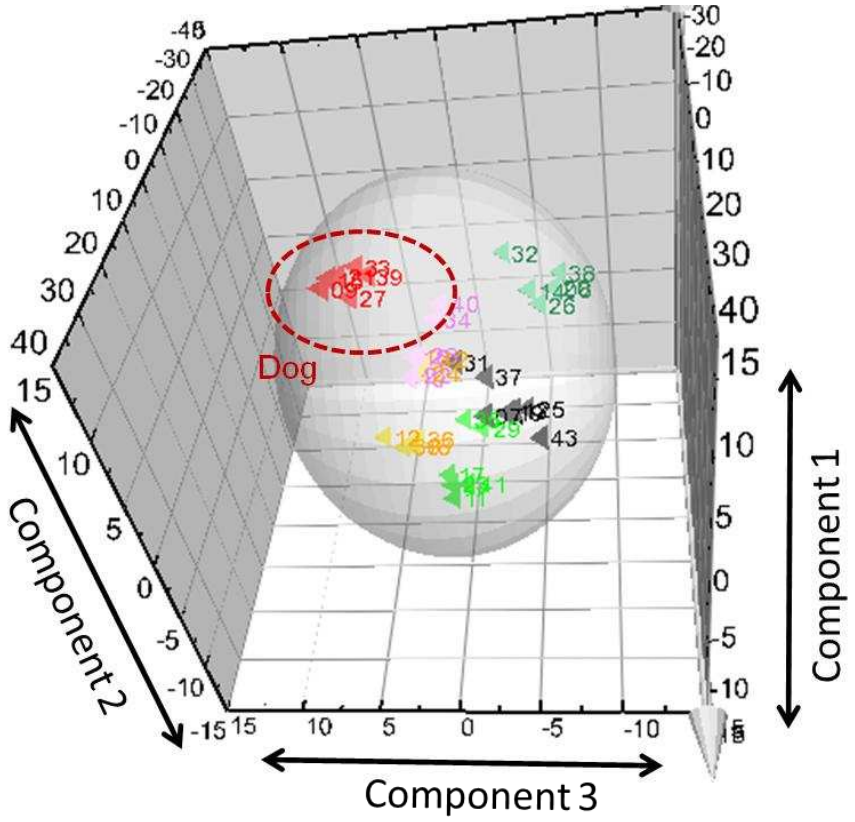


Figure 7-11; 3 dimensional plot of PLS components 1, 2 and 3 showing separation of the cluster of dog samples

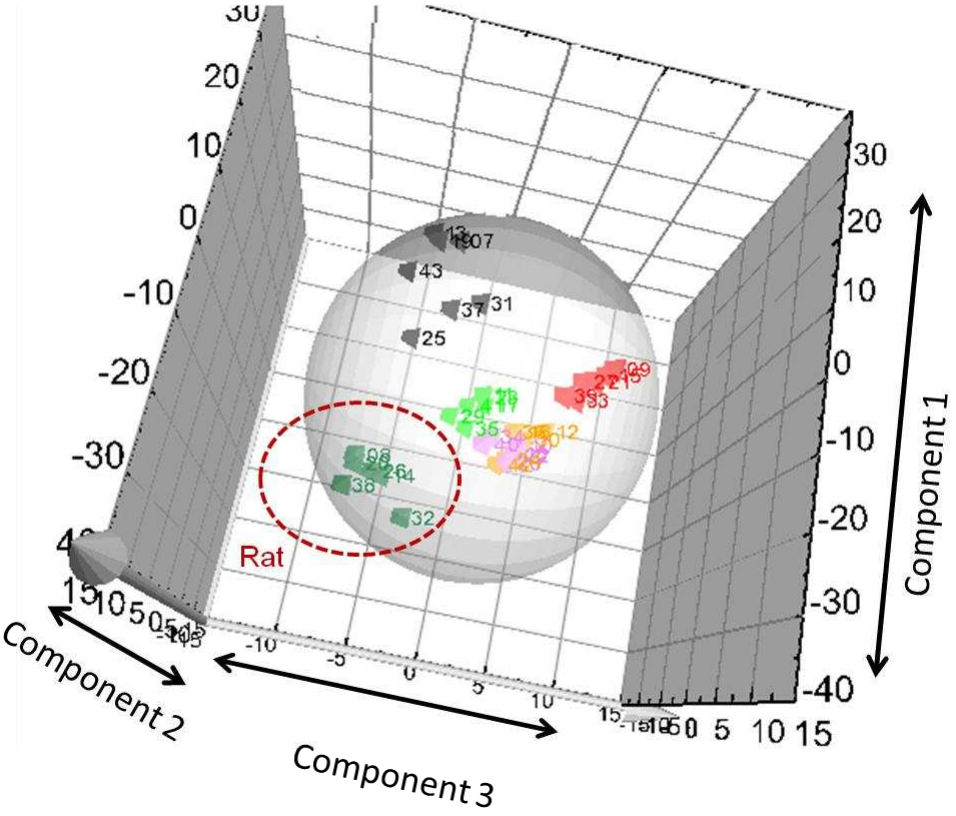


Figure 7-12; 3 dimensional plot of PLS components 1, 2 and 3 showing separation of the cluster of rat samples

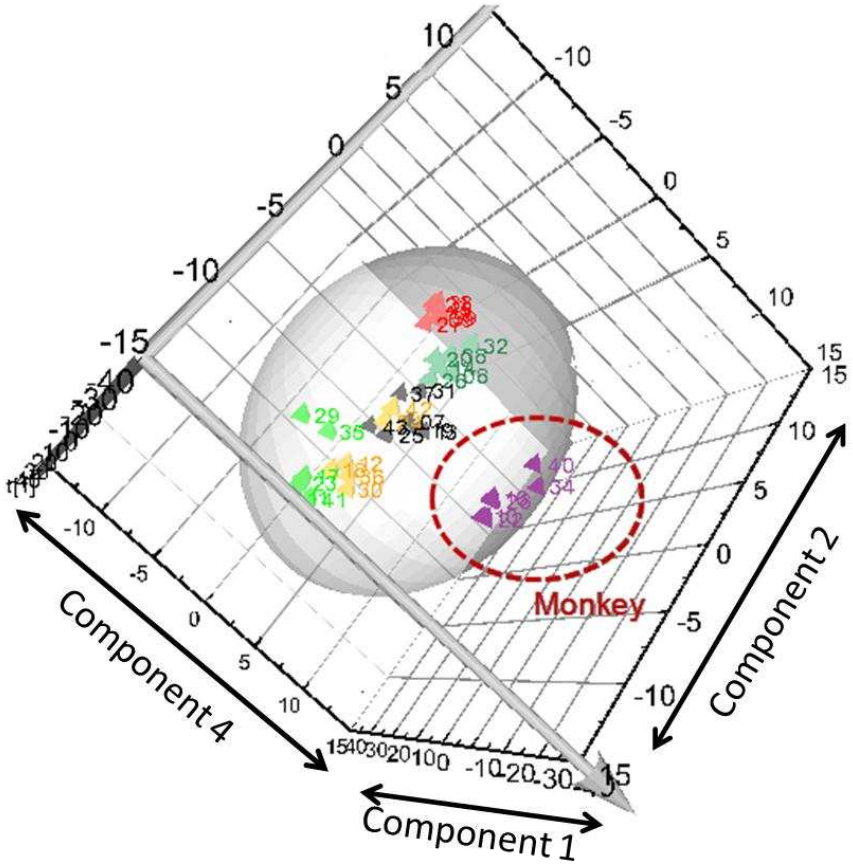


Figure 7-13; 3 dimensional plot of PLS components 1, 2 and 4 showing separation of the cluster of monkey samples

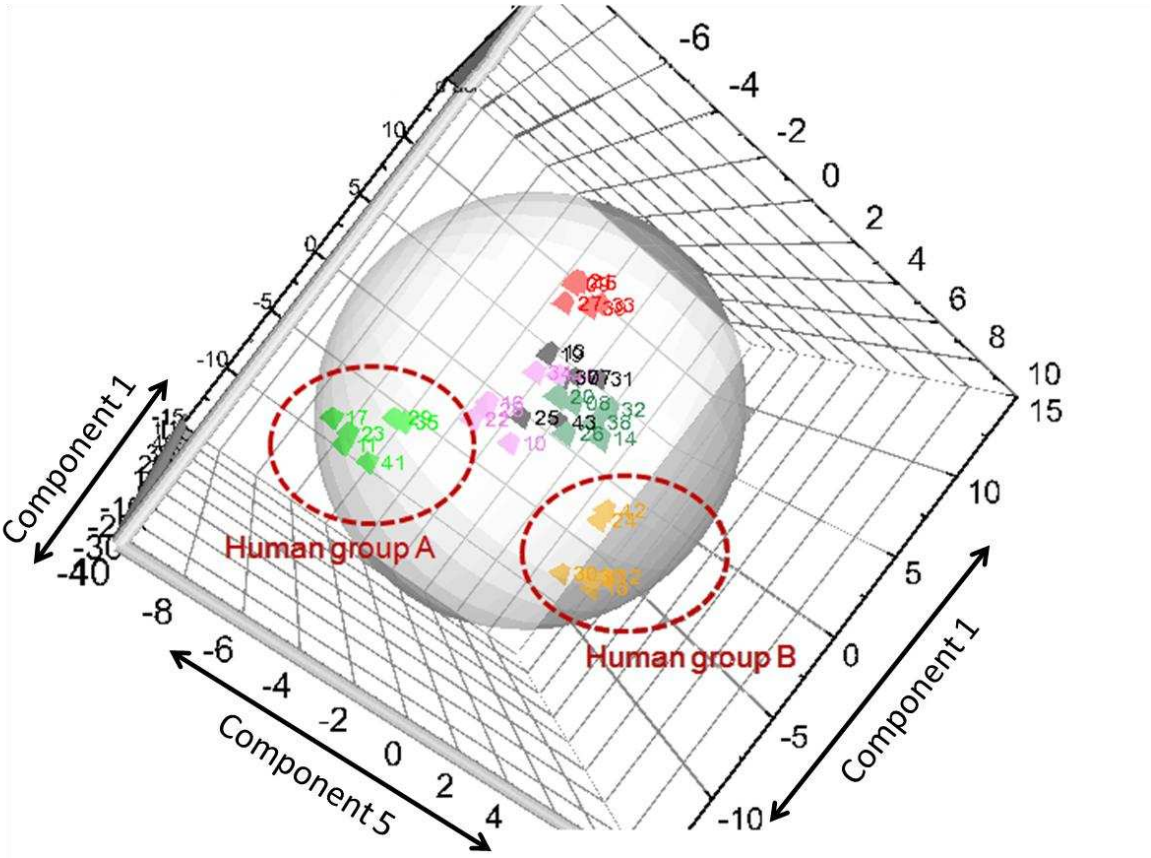


Figure 7-14; 3 dimensional plot of PLS components 1, 2 and 5 showing separation of the cluster of individual human samples of 2 different ethnic origins

	Precursor ion / Retention time (seconds)	Variable Importance	Confidence Interval
1	**793/376**	1.91	1.44
2	**607.5/345**	1.81	1.12
3	**520.3/133**	1.72	0.322
4	**753.6/334**	1.62	1.05
5	**793.8/508**	1.61	1.32
6	**759.1/377**	1.60	1.15
7	**510.4/159**	1.58	1.09
8	**601.5/90**	1.58	1.17
9	**512.4/188**	1.57	1.33
10	**765.9/462**	1.53	1.05
11	**787.8/597**	1.50	1.20
12	**538.3/205**	1.50	0.758
13	**690.7/417**	1.49	0.914
14	**703.9/393**	1.49	1.15
15	**771.7/558**	1.47	1.11
16	**574.4/189**	1.45	1.22
17	**799.8/391**	1.45	1.04
18	**565.5/303**	1.44	0.785
19	**773.8/553**	1.42	0.719
20	**827.1/385**	1.42	0.366
21	**568.3/103**	1.41	1.00
22	**544.6/133**	1.41	0.566
23	**587.2/106**	1.41	1.17
24	**481.5/183**	1.40	1.18
25	**567.4/102**	1.39	0.834

	Precursor ion / Retention time (seconds)	Variable Importance	Confidence Interval
26	**571.4/164**	1.37	0.595
27	**823.9/583**	1.37	0.632
28	**777/642**	1.37	0.438
29	**551.5/231**	1.36	0.485
30	**819.8/419**	1.35	1.02
31	**784.5/493**	1.34	0.881
32	**548.8/171**	1.34	0.781
33	**674.7/338**	1.33	1.29
34	**522.7/153**	1.33	0.645
35	**728.9/409**	1.32	1.02
36	**802.1/382**	1.32	0.547
37	**471.5/128**	1.31	0.572
38	**823/529**	1.30	1.23
39	**521.4/153**	1.30	0.910
40	**585.9/101**	1.30	1.06
41	**545.4/154**	1.29	0.702
42	**559.2/91**	1.29	0.791
43	**763.1/578**	1.28	0.841
44	**623.6/257**	1.28	0.959
45	**788.4/608**	1.28	0.950
46	**799/524**	1.27	0.822
47	**855.1/430**	1.27	0.969
48	**609.7/404**	1.27	0.912
49	**702.8/393**	1.27	0.915
50	**807/441**	1.26	0.940

Table 7-2; List of the most important variables (precursor/retention time)

7.2.1 Validation of the Separation Model

To validate the model described by using an external test dataset, a series of plasma samples were analysed for predictive comparison with the model dataset containing n = 2 unique samples from each of the test groups. These samples were concurrently analysed but did not contribute to the separation model. As the graphs in Figure 7-10 to Figure 7-14 demonstrate, while each component sample type may be visually separated in 2 or 3 dimensions from the entire group, a common model would require separation in more than 3 dimensions. Figure 7-7 shows that the SIMCA prediction identified 9 PLS components to represent separation of the entire dataset. To demonstrate the separation of 2 plasma types (rat and dog) which can be visually separated on a plot of PLS components 1 and 2, the test set was compared to the training by plotting each set of data on super imposable elliptical plots (Figure 7-15). Test samples from rat and dog show the same orientation on the plot in both training and test datasets.

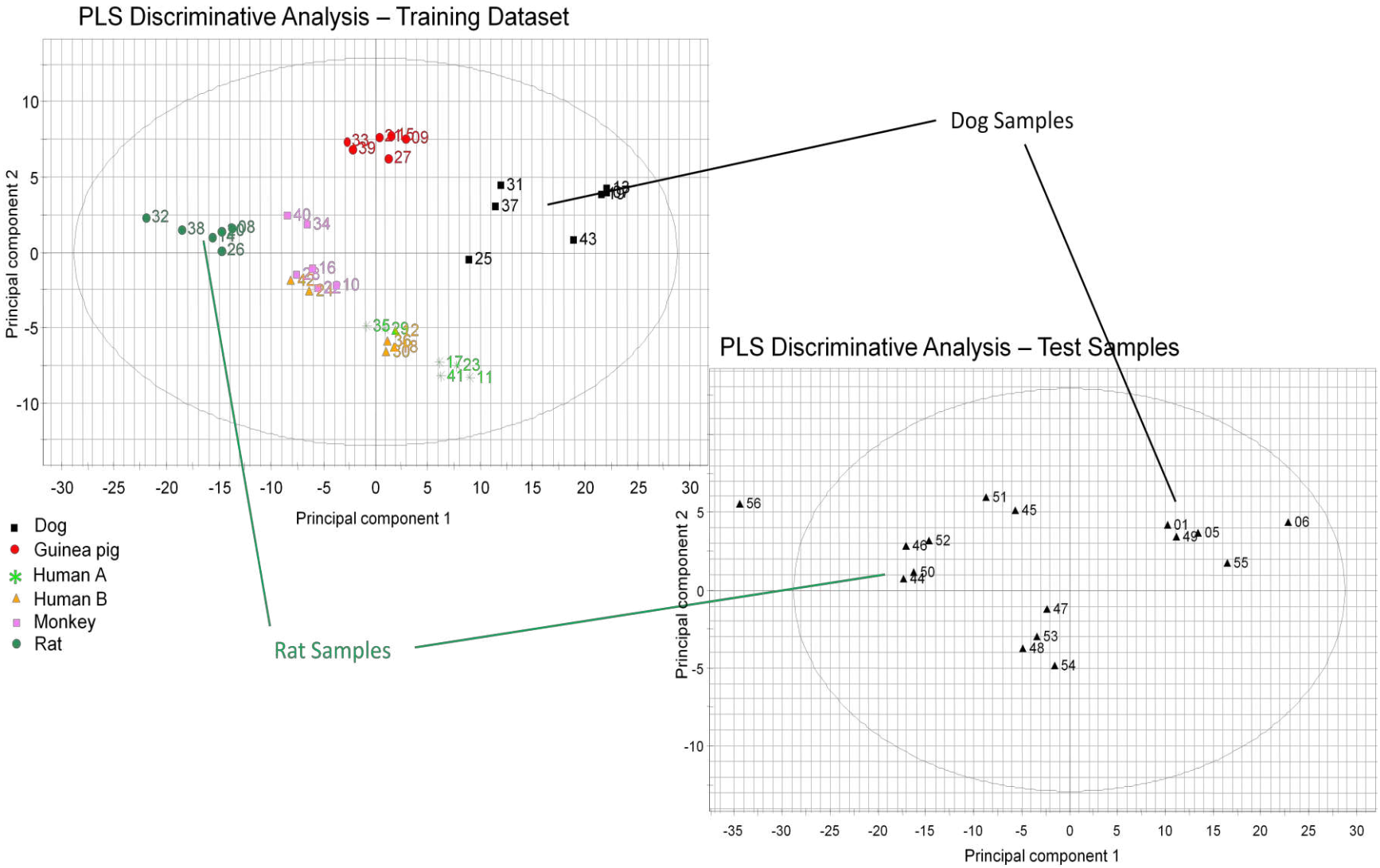


Figure 7-15; Super imposable elliptical plots of PLS component 1 against PLS component 2 showing the same orientation of external test samples

Using SIMCA software it was possible to use the entire 9 PLS components to predict the identity of the external samples unknown to the PLS model. This is done by comparing data from each test sample to each specified group of variables (species) using the model algorithm to present a numerical indication of probability that the test data point belongs to each group. The results of this test are reported in Table 7-3. The data shows a correct prediction of dog, guinea pig and rat samples with scores greater than 0.6 and significantly greater than scoring from other groups and indicated as a good fit to the model while for the primate samples from two different human ethnic groups and monkey samples, the identities are suggested but scores show insufficient magnitude or clear separation from other groups. A species difference in phospholipid distribution is demonstrated while accurate prediction would require the construction of a model with fewer variables which could be fitted more discriminatively.

ID	Actual ID	Predicted probability						Predicted ID
		Dog	Guinea Pig	Human A	Human B	Monkey	Rat	
1	Dog	0.697	0.242	0.0262	0.173	-0.0856	-0.0522	Dog
2	Dog	0.769	0.116	0.167	0.035	-0.0724	-0.0146	Dog
3	Guinea Pig	-0.0318	0.674	-0.0113	0.124	0.0872	0.158	Guinea Pig
4	Guinea Pig	-0.131	0.759	-0.075	0.0321	0.180	0.235	Guinea Pig
5	Human A	0.167	0.0981	0.211	0.409	-0.0363	0.152	
6	Human A	0.191	-0.0812	0.301	0.421	0.0518	0.117	
7	Human B	-0.148	0.203	0.264	0.632	-0.0453	0.0942	(Human B)
8	Human B	0.0170	-0.0578	0.127	0.544	0.250	0.119	(Human B)
9	Monkey	-0.0914	0.184	-0.0898	0.142	0.305	0.550	
10	Monkey	-0.0484	0.250	-0.123	0.0188	0.538	0.365	(Monkey)
11	Rat	-0.0872	-0.0303	-0.0557	0.131	0.390	0.652	Rat
12	Rat	-0.0917	0.102	0.0113	-0.0197	0.263	0.735	Rat

Table 7-3; SIMCA PLS data external dataset prediction

7.3 Variability in Phospholipids from Human Subjects

7.3.1 Dietary State

To investigate the influence of dietary fat state on phospholipid distribution, lithium heparinised plasma from n = 18 individual male and female donors from 3 different ethnic groups following overnight fasting and n = 18 donors with visibly lipemic plasma were analysed by the method described in chapter 8 monitoring precursors of m/z = 184 Da. Lipemic plasma is typically milky in colour and turgid due to elevated concentrations of

circulating lipids particularly triglycerides at concentrations greater than 800 mg/dL, [216] the normal triglyceride concentration range is 40 to 200 mg/dL. [8] Elevation usually occurs temporarily following meals (alimentary lipemia) before clearance to unesterified fatty acids [217] but also occurs chronically as a result of some medical conditions such as diabetes.

The profiles obtained indicate some visible differences as demonstrated in Figure 7-16. The ion transition selected as the most clearly differing peak at 2.93 minutes (560.8 →184 Da) was extracted and used to construct an overlaid mass chromatogram and presented in Figure 7-17 (constructed using from samples from Caucasian individuals to eliminate ethnic differences). The maximum intensity of the mass chromatogram for this ion transition is approximately 50% greater in lipemic than fasted individuals.

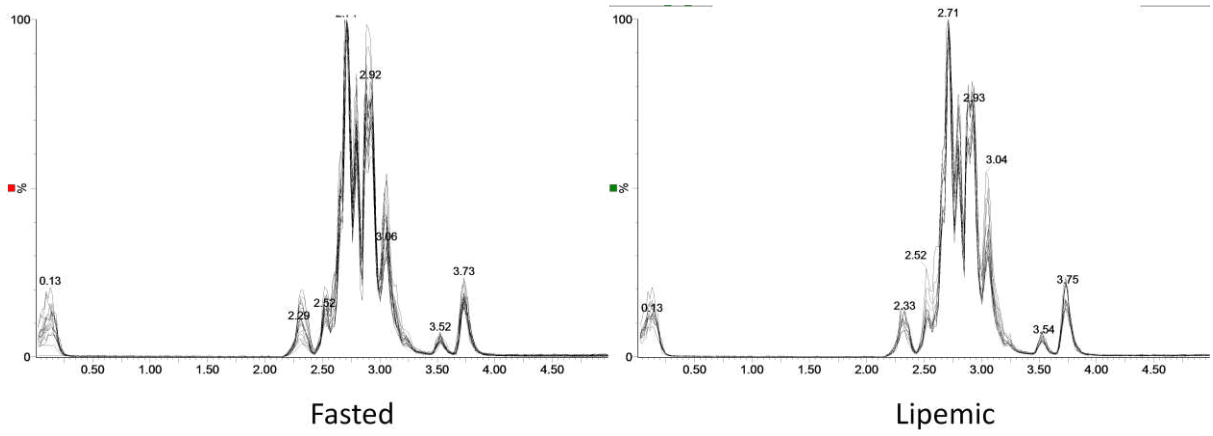


Figure 7-16; Overlaid phospholipid profile in plasma samples from fasted and lipemic volunteers of all ethnic groups (n=18)

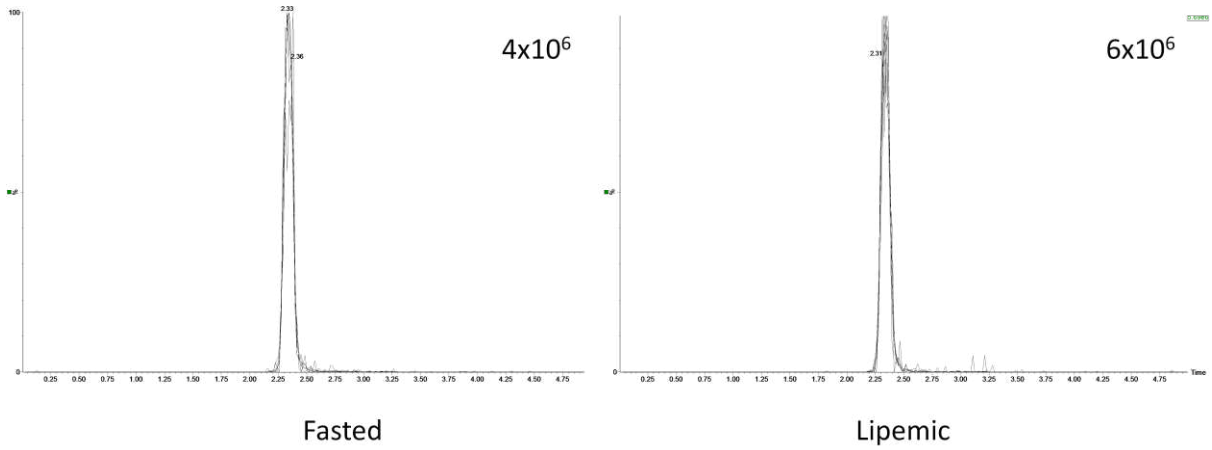


Figure 7-17; Overlaid 560.8>184 transition mass chromatogram in plasma samples from fasted and lipemic Caucasian volunteers (n=6)

The results of PLS-DA analysis of the derived data are presented in Figure 7-18. The data shows a separation between datasets, which may be an effect of incorporation of dietary fatty acids. Since clinical pharmacokinetic studies are conducted across day long time periods incorporating daily food intake and further studies are conducted to compare the bioequivalence of drug exposure in fed and fasted volunteers to characterise food effects, the potential variability in phospholipid distribution in response to diet is demonstrated.

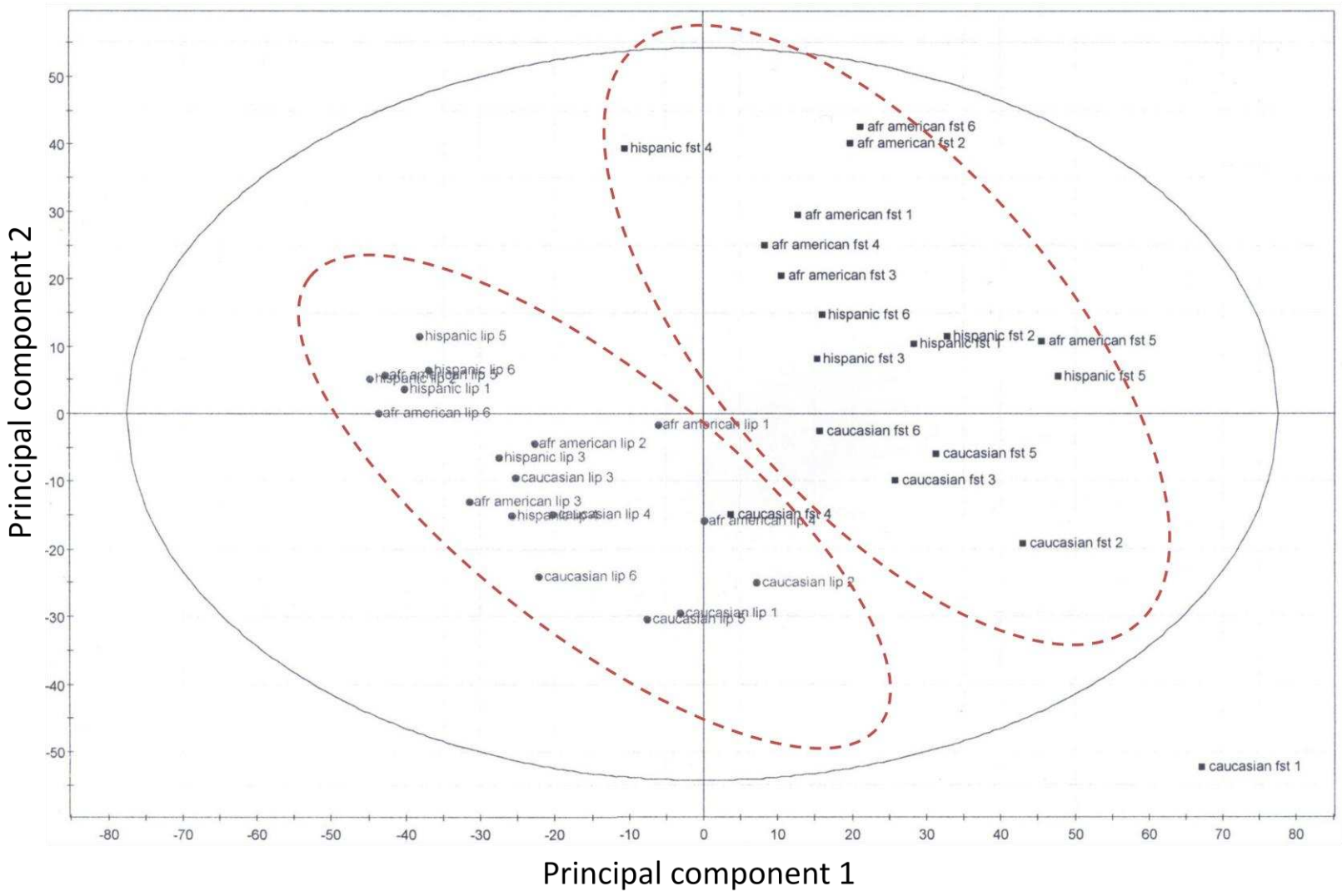


Figure 7-18; Separation of lipemic and fasted groups by PLS regression

7.3.2 Variability in Phospholipids from Different Ethnic Groups

In assessing the effect of diversity in human population, a definable determinant of diversity is subject ethnicity. Reported studies have identified differences in the distribution of various lipid molecular species among different populations [218- 220] relating their observations to nutritional or epidemiological investigations particularly in relation to cardiovascular health. Previous observations of the effect of dietary lipids on phospholipid distribution might indicate that extrinsic cultural or regional factors may influence this; however intrinsic genetic factors are also associated with metabolic differences. [221] Ethnic differences are additionally associated with predisposition to autoimmune conditions effecting endogenous phospholipids.

The importance of ethnic group as a factor influencing pharmacokinetic parameters is acknowledged by the publication of ICH/EMEA regulatory guidance on the evaluation of ethnic factors on drug safety and efficacy. [222] Characterisation of pharmacokinetics for new drug registration must be conducted in populations appropriate to the geographical region when the new drug is to be registered. This is notably the case for drugs developed in early clinical studies in Europe and North America involving Caucasian, Hispanic and African American volunteers and registered for use in Japan for prescription to Japanese patients. Such pharmacokinetic bridging studies intended to demonstrate the validity of extrapolation of clinical study data regarding safety and efficacy will also be conducted reciprocally where drugs registered in Europe and North America have been extensively developed in China or India. The guidance describes that intrinsic and extrinsic factors may influence the ethnic effect on pharmacokinetics so the ethnicity of the volunteers is as significant as the geographical location of the study site. While the FDA also provides guidance on the identification of the ethnic origin of clinical trial subjects, [223] no consideration is yet given to the demographics of control matrix donors.

To investigate the potential ethnic difference between phospholipids, lithium heparinised plasma samples from n = 12 male volunteers (fed and fasted) of Caucasian, Hispanic and African American origin were analysed using the initial method described in 7.3.1. The typical profiles obtained were indistinguishable by visual comparison as shown in Figure 7-19. The summed precursor ion mass spectra of representative individuals represented in

Figure 7-20 does not show the distinguishable differences seen in the inter species comparisons seen in Figure 7-3. The data set was subjected to PLS analysis and separation observed (Figure 7-21).

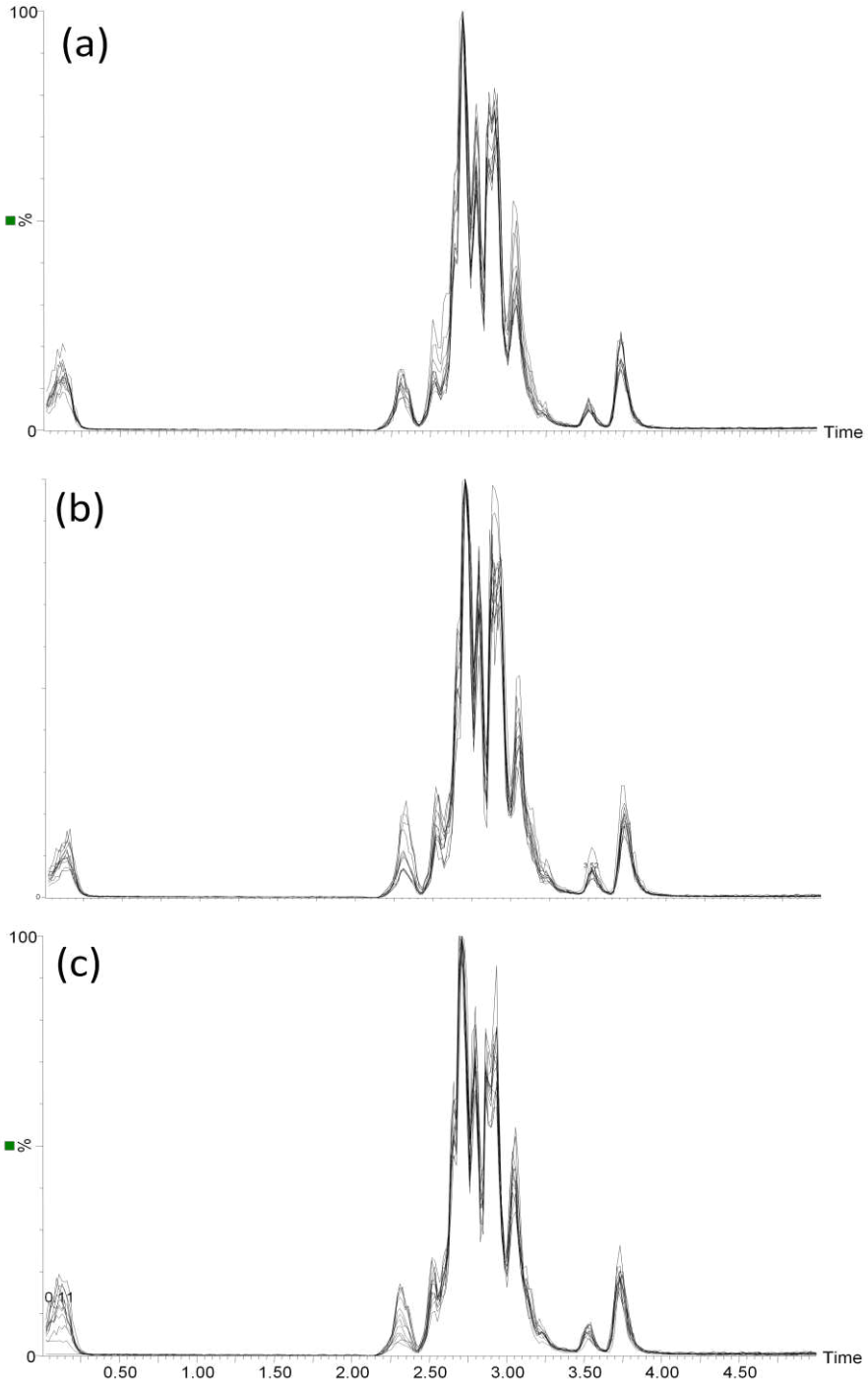


Figure 7-19; Phospholipid profile (total ion count precursors of m/z 184 Da) of (a) African American, (b) Hispanic and (c) Caucasian individuals (n = 12, overlaid)

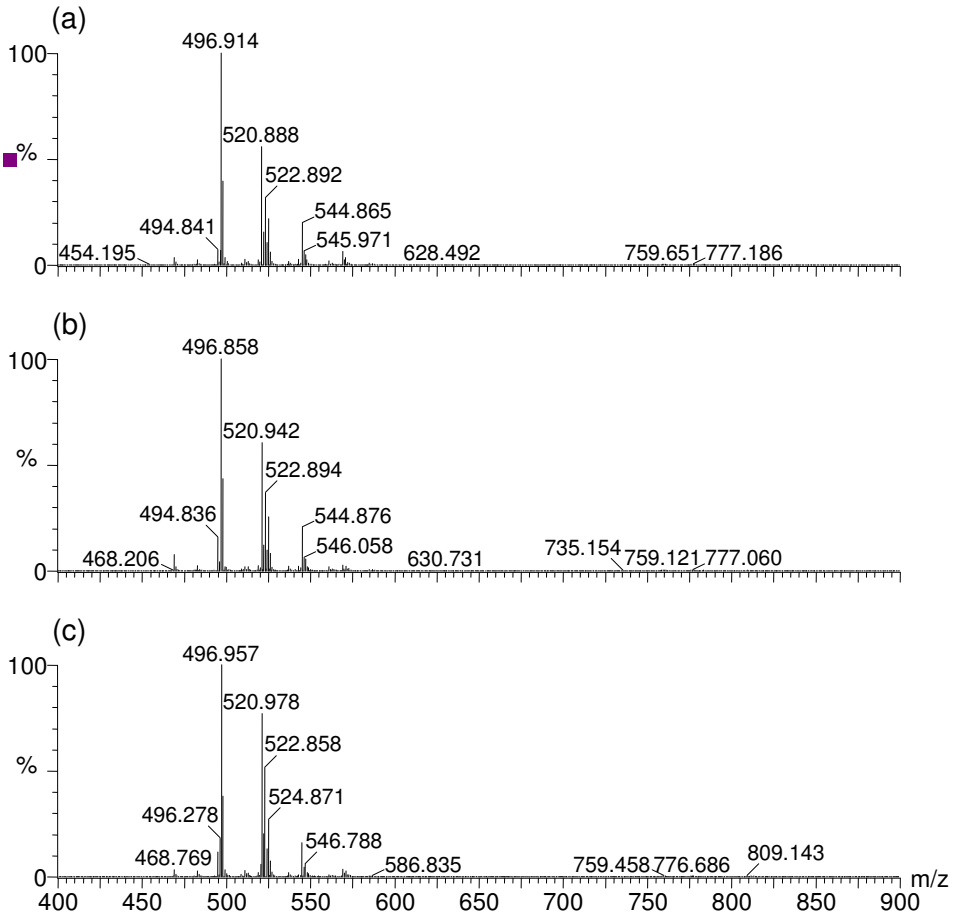


Figure 7-20; Summed ions approximately 120 to 240 seconds from (a) African American, (b) Hispanic and (c) Caucasian individuals

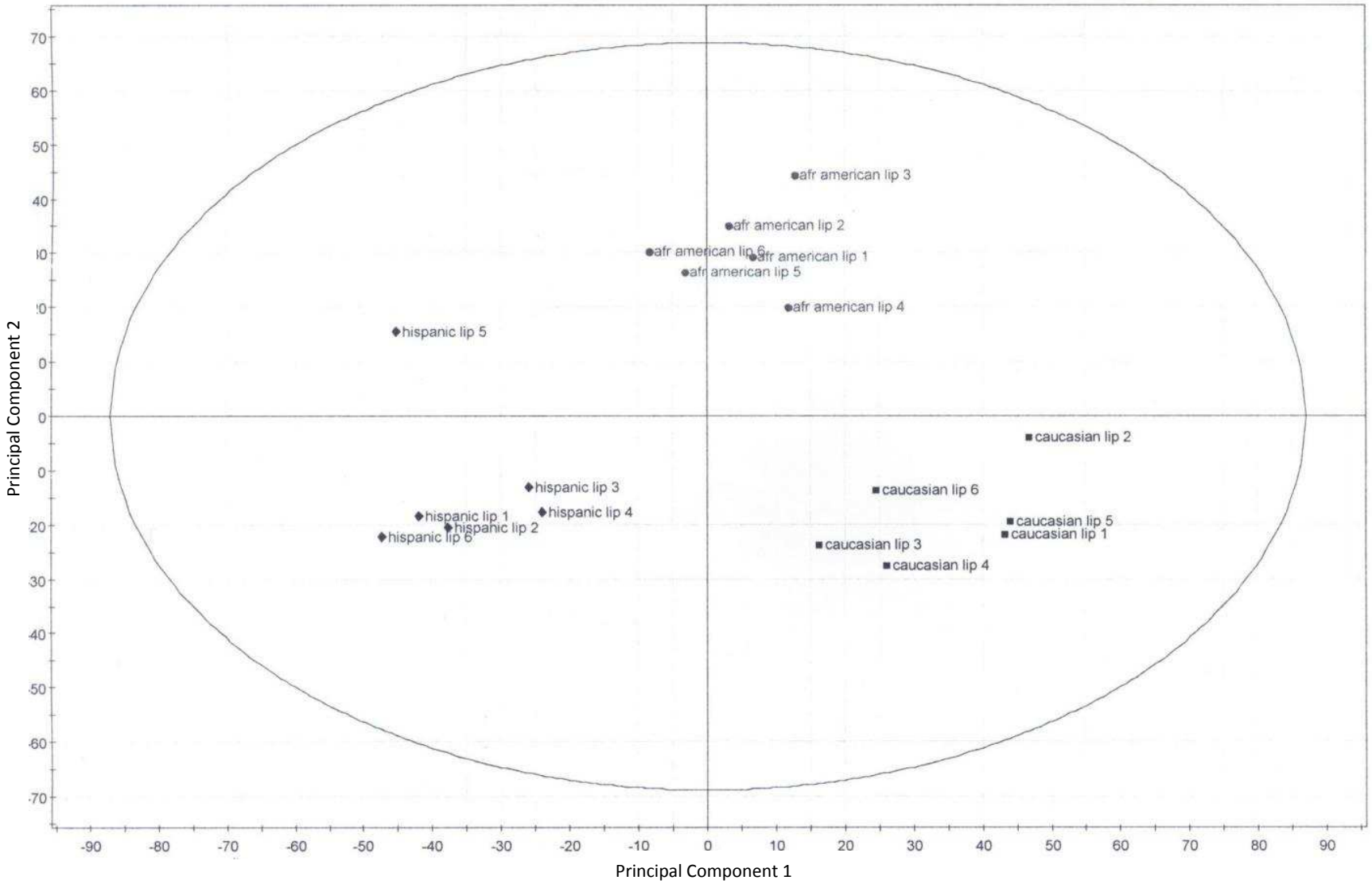


Figure 7-21; Separation of phospholipid profiles of individuals from 3 ethnic groups by PLS regression (lipemic samples)

Following observation of this separation and in consideration of the implementation of the EMEA regulatory guidance [222] by Japanese regulatory authorities, it was proposed to repeat the analysis incorporating samples from Japanese volunteers. The experiments were performed using the expanded separation method described in Table 7-1 by analysing n = 6 K_2EDTA derived plasma samples obtained from male volunteers of Japanese origin and male and female volunteers of African American and Caucasian origin. The PLS analysis is presented in Figure 7-22. The separation appears to indicate a greater difference between Japanese individuals and the remaining groups. This is attributed in part to geographical difference between donors, the Japanese donors living in Kawagoe, Japan while all other samples were obtained from Virginia, USA.

These differences may contribute to extrinsic differences between samples, particularly dietary, but as such are illustrative of the additional diversity of sample origins from multinational sources. The investigation into ethnic differences with a limited available dataset suggested that it was possible to differentiate between groups using PLS regression; however, the model obtained was not predictive.

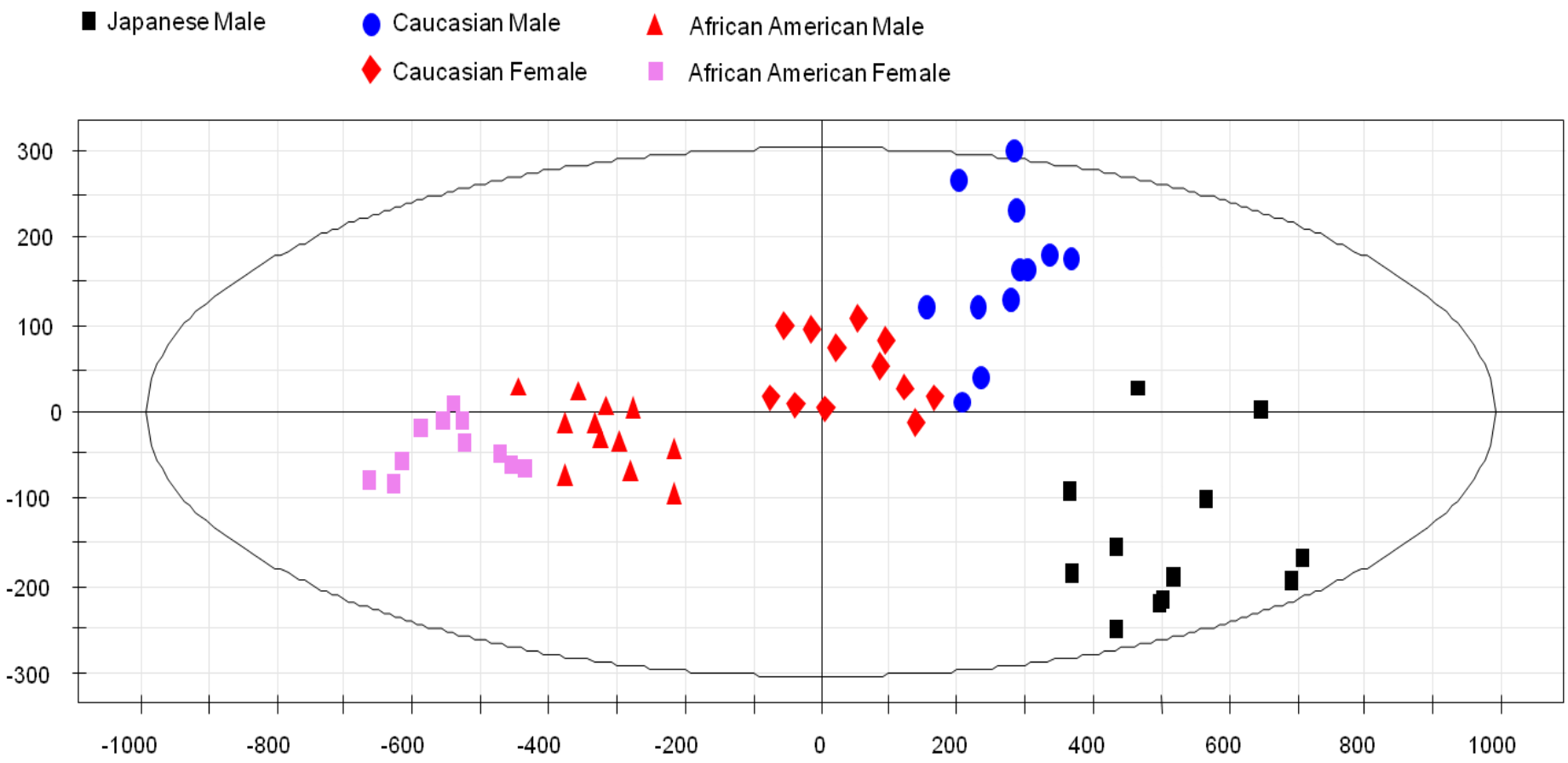


Figure 7-22; Separation of phospholipid profiles of individuals from individuals of Japanese Male, Caucasian Male and Female and African American Male and Female origin by PLS regression

7.4 Phospholipid Investigation Conclusions

The distribution of membrane phospholipids in animal and human plasma samples was investigated following the development of a Turboflow LC-MS/MS method and application together with a mass spectrometric analysis method with selectivity for the detection of the most abundant group of membrane phospholipids (phosphotidylcholine and sphingomyelin). Data was analysed using partial least squares regression discriminative analysis, and separation was demonstrated between plasma from groups of individual animals indicating differences in the distribution of membrane phospholipids between species. The observations were verified by the analysis of 'unknown' samples which were correctly identified by superimposing plots and by a probability calculation conducted using SIMCA software.

Application of the experiments to plasma samples from individuals of different human ethnic groups showed separation but no predictive data verification was obtained. Samples from fasted individuals were compared with lipemic plasma samples and differences noted both by mass chromatogram comparison and by PLS-DA analysis. The differences observed in the distribution of phospholipids circulating in sources of test plasma between species support the proposal that inter-species differences in phospholipid distribution are responsible for the alternative signal responses from analyte and deuterated analogue observed in chapter 6.

8 Results and Discussion – SARY; Preliminary Work

8.1 Introduction

X-tert-butylurea, (subsequently referred to as SARY; Figure 8-1) is new chemical entity in pharmaceutical development by Sanofi-Aventis. [224]

Figure 8-1; Partial structure of SARY

SARY has a molecular weight = 539.4 and a pKa value of 1.2. The calculated log D of the compound (Figure 8-2) shows that its distribution coefficient is largely unaffected by pH within the working range of most silica based HPLC columns without loss of robustness caused by column damage.

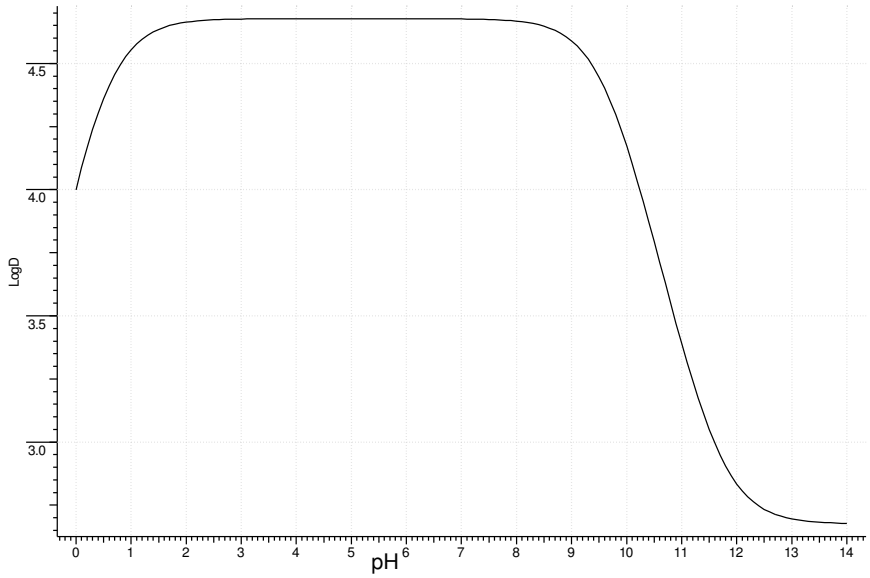


Figure 8-2; Relationship between logD and pH for SARY

A stable isotope analogue was available for use prepared by substitution of the ^{1}H hydrogen atoms for ^{2}H deuterium on all three methyl groups in the tert-butyl group. This resulted in a heavy labelled compound with a molecular mass of 548.4. The difference of 9 mass units

from the unlabelled analyte was sufficient to prevent cross-talk in mass spectrometer response as a result of the isotopic distribution of the two chlorine atoms in the structure. The nonadeuterated analogue was therefore sufficiently discriminative for use as an internal standard for discriminative mass chromatogram quantification.

8.2 Sample Preparation and Preliminary Analytical Procedure

To facilitate method optimisation, analytical samples were prepared within the concentration range appropriate to the predicted range of study sample concentrations calculated by the pharmacokineticist at Sanofi-Aventis. A preliminary analytical system was assembled as described in section 6.2 with chromatographic conditions subjected to optimisation.

Preparation of standard solutions

Stock standard solutions (500 µg/mL) were prepared in DMSO X-tert-butylurea and stored under ambient conditions. The stock standards were further diluted with DMSO to yield standard solutions of 2, 5, 10, 25, 50, 100, 250 µg/mL. Internal standard solution of X-tert-nonadeuterobutylurea (20 µg/mL) was prepared in DMSO.

Preparation of human plasma analytical samples

Plasma calibration samples were prepared by mixing control human plasma (4.95 mL) with an aliquot (50 µL) of the SARY standard solutions to give concentrations of 20, 50, 100, 250, 500, 1000, 2500, 5000 ng/mL. Analytical samples were processed immediately or stored frozen (-20 $\pm$ 5°C). A second set of validation test standard samples were prepared in control human plasma at 20, 50, 500, 5000 ng/mL. Working internal standard sample (67 ng/mL) was prepared by mixing internal standard solution with K^+EDTA derived control human plasma.

Preparation of samples for analysis

Each plasma sample (50 µL) was added to a 96 deep well polypropylene microtitre plate and mixed with 150 µL of internal standard ([$^2H_9$]-SARY in plasma). The plate was sealed with a pierceable square well polypropylene sealing cap and the plate mixed for 10 minutes using a microtitre plate mixed (Micromix 5, Llanberis, UK) with a frequency alternating between 20 and 25 Hz set to maximum amplitude, then centrifuged 4000 rpm (2800 rcf) under ambient

conditions for 10 minutes on an Eppendorf 5810R centrifuge (Eppendorf, Cambridge) to enable maximum contact between small volume components. The samples were mixed for 10 minutes on a vortex plate mixer and then centrifuged for a further 10 minutes (4000 rpm; ambient temperature) to remove suspended particulates from the plasma sample to prevent needle blocking on the autosampler or transfer of particulates to the extraction column frits. A mass spectrometry procedure was defined by infusing a standard solution prepared in acetonitrile via syringe pump at approximately 5 µL/sec directly into the instrument and tuning acquisition parameters to obtain the values listed in the Table 6-2 operated using electrospray ionisation. The precursor and product ion mass spectra obtained are shown in Figure 8-3 and Figure 8-4. A precursor ion is shown at m/z = 539 Da. Fragmentation of the peak at m/z = 539 Da reveals 4 main product ions. The greatest intensity product ion of m/z = 440 Da was selected as the product ion for use in the MS/MS transition to generate a signal for quantitative chromatographic analysis ie, m/z = 539>440 Da.

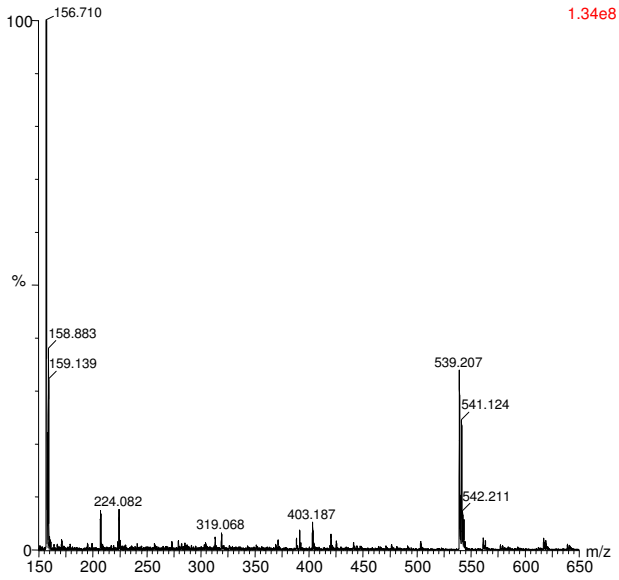


Figure 8-3; Mass spectrum of SARY

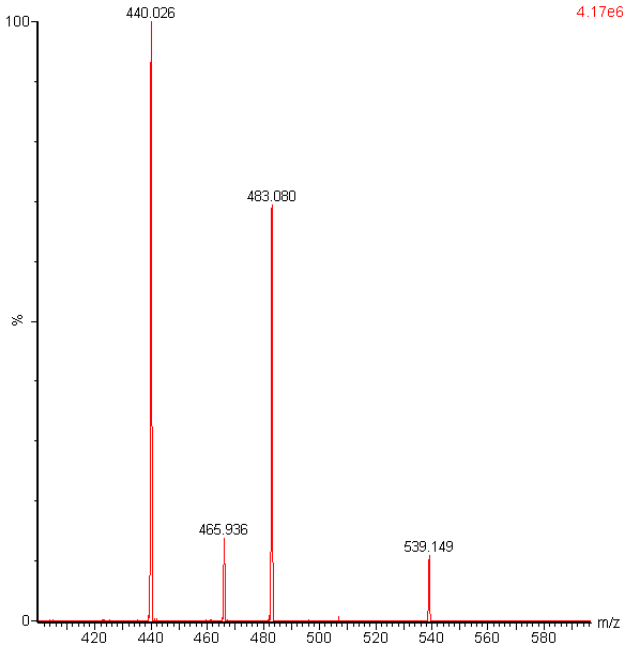


Figure 8-4; Product ion mass spectrum of SARY

8.3 Extraction Column Recovery

8.3.1 Drug Recovery

To maximise the recovery of drug from unmodified plasma using a simplified column switching method, recovery was assessed by loading a 0.5 mm i.d. Turboflow column for 30 seconds at 1.25 ml/min with 20 mM pH 3.0 ammonium formate and injecting an aliquot (50 µL) of control human plasma into the flow. Following elution of bulk macromolecules through the column, flow was reversed and an elution mobile phase at 0.75 mL/min applied containing a variable amount of organic modifier to assess recovery. The eluent was split with a ratio of approximately 1:1 v/v and directed to the mass spectrometer where the transition m/z = 539.1 > 440 Da was monitored. The experimental sequence was repeated in replicates of 3 using both methanol and acetonitrile as the organic component and C2, C8, C18 and Cyclone (reversed phase polymer) extraction columns were tested. The results of the recovery experiments in each column using methanol and acetonitrile in the elution mobile phase are represented in Figure 8-5 and Figure 8-6.

Experimental Method Parameters	MS Acquire time	5 minutes	
	Type	Multiple Reaction Monitoring	
	Ion Mode	ESI +ve	
	Channel 1	Precursor m/z	539.1 Da
		Product m/z	440 Da
		Span	0
		Dwell (sec)	0.3
		Delay (sec)	0.03
	Channel 2	Precursor m/z	548.1 Da
		Product m/z	440 Da
		Span	0
		Dwell (sec)	0.3
		Delay (sec)	0.03
ESI Source Parameters	Source Temperature (ºC)	120	
	Desolvation Temperature (ºC)	350	
	Collision Gas Pressure (MBar)	1×10^{-3}	
	Cone Gas Flow (L/h)	55	
	Desolvation Gas (L/h)	900	
	Cone Voltage (V)	50	
	Capiliary Voltage (kV)	1	
	RF Lens 1 (V)	20	
	Aperture (V)	1	
	RF Lens 2 (V)	0	
Typical Analyser Parameters	LM1 Resolution	14	
	LM2 Resolution	14	
	Ion Energy 1	0.5	
	Entrance	0	
	Collision	32	
	Exit	1.0	
	LM1 Resolution	14	
	LM2 Resolution	14	
	Ion Energy 1	0.8	
	Multiplier	650	

Table 8-1; Mass spectrometer operating parameters

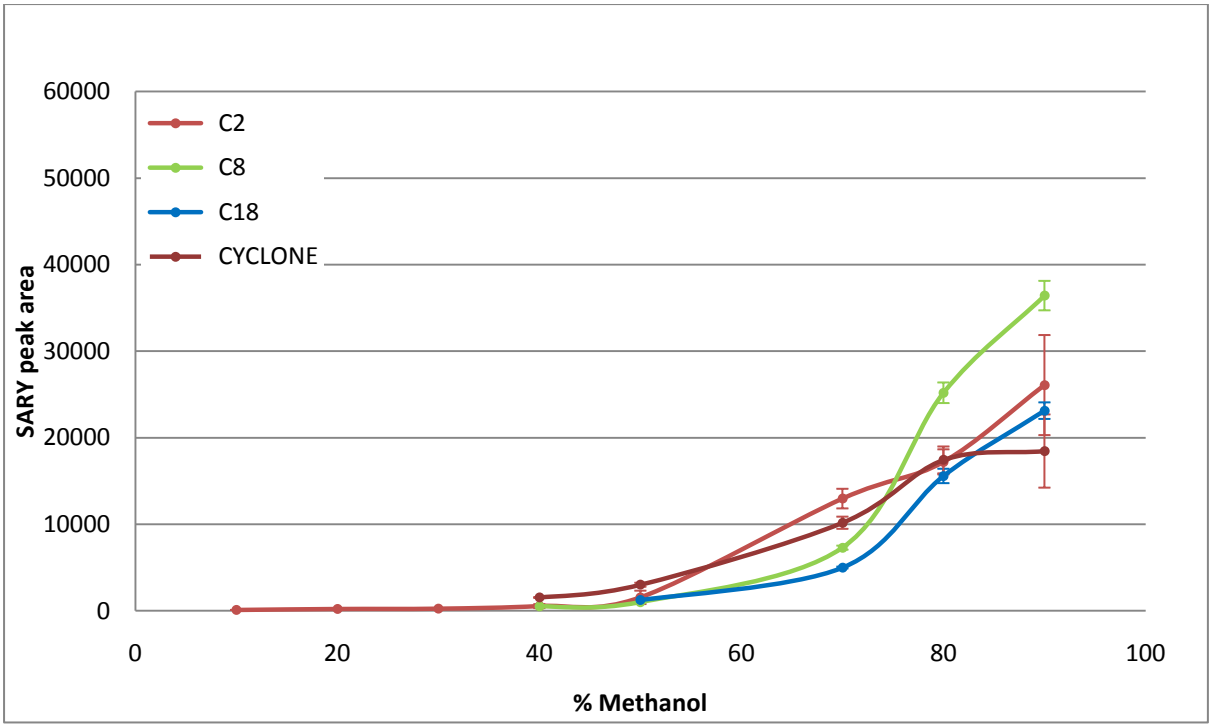


Figure 8-5; Recovery of SARY from Turboflow columns using variable methanol content; peak area (±sd)

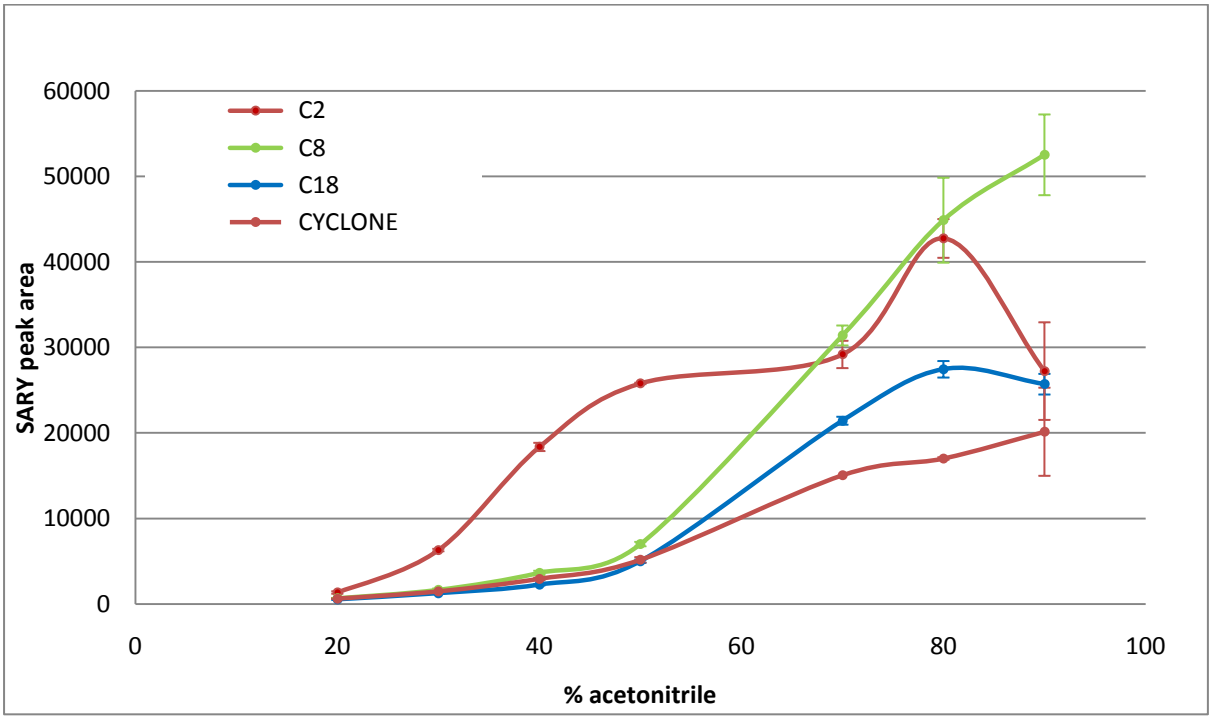


Figure 8-6; Recovery of SARY from Turboflow columns using variable acetonitrile content; peak area (±sd)

The data shows that recovery increased proportionately with solvent content of the elution mobile phase with the exception of the C2 column in the presence of high proportions of acetonitrile. It is proposed that the reduced carbon loading of the C2 modified column and

accessibility of surface silanol activity induced the contribution of a hydrophilic interaction retention mechanism in the presence of a highly organic mobile phase. While optimum recovery via both elution solvents was obtained using a C8 column, peak areas were greater by acetonitrile rather than methanol.

8.3.2 Phospholipid Recovery

Using the methodology described in 8.3.1, the experiments were repeated to monitor the recovery of 4 probe phospholipid species. The species monitored were m/z= 496>184, 704>184, 761>184 and 787>184 Da. These experiments were conducted using acetonitrile as the elution mobile phase as selected above and all experiments were conducted in replicates of 3. The results of the experiments are presented in Figure 8-7 to Figure 8-12.

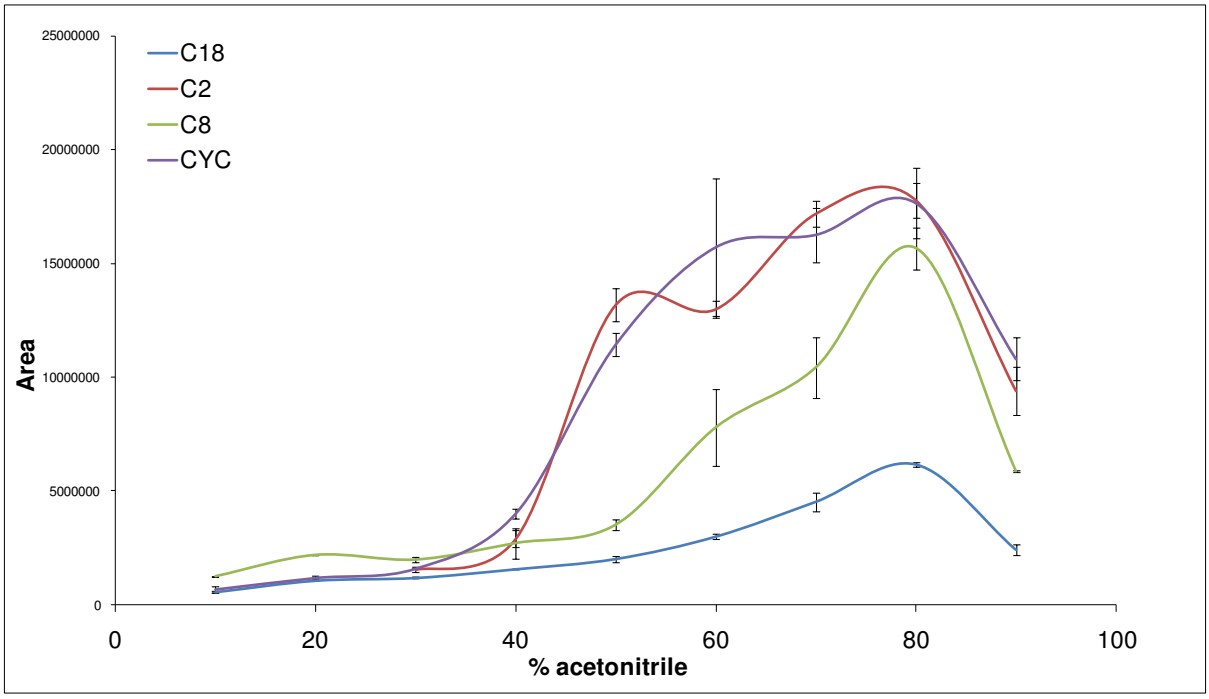


Figure 8-7; Recovery of phospholipid 704>184 from Turboflow columns using variable acetonitrile content

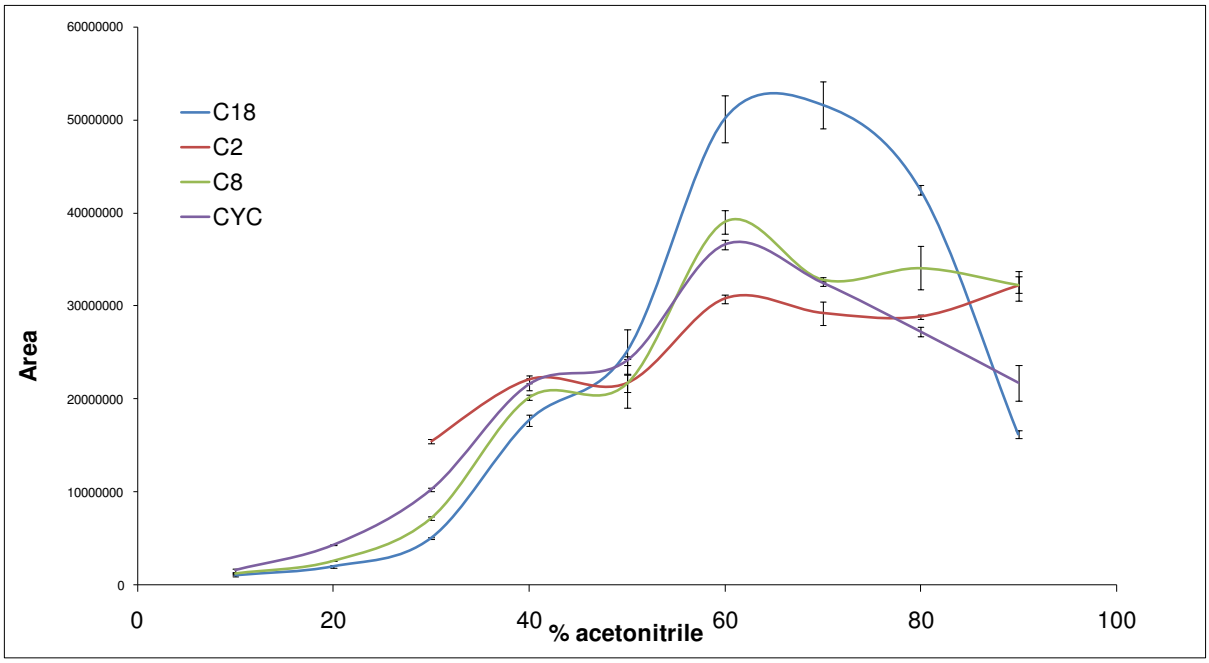


Figure 8-8; Recovery of phospholipid 496>184 from Turboflow columns using variable acetonitrile content

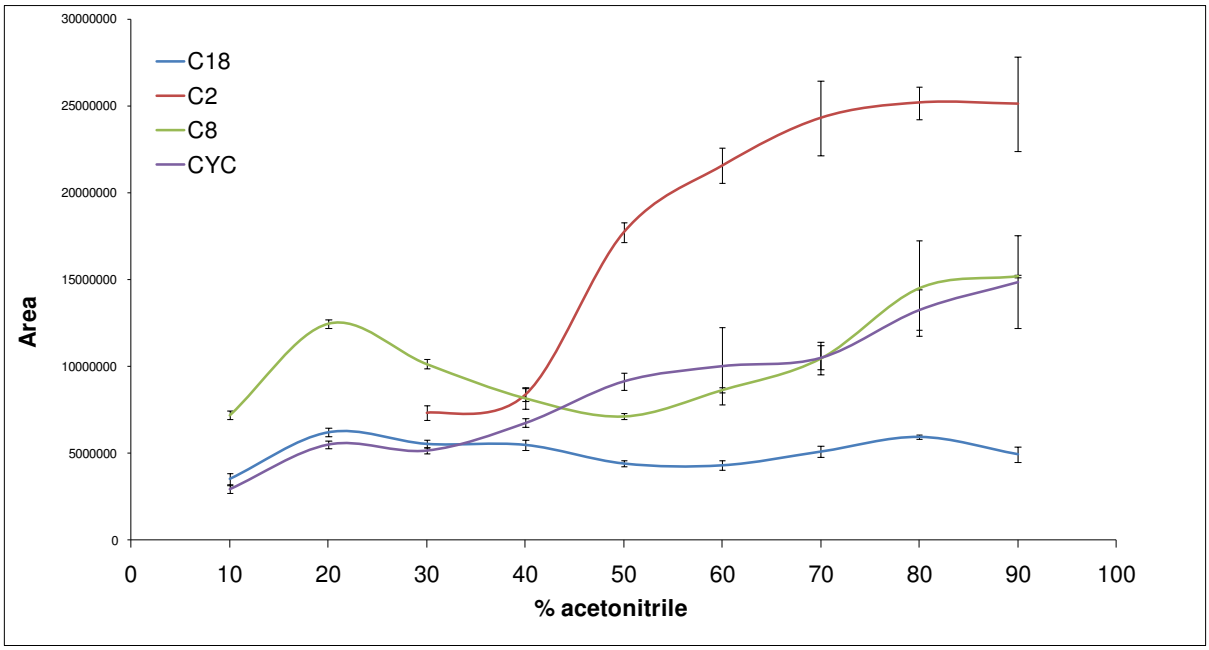


Figure 8-9; Recovery of phospholipid 761>184 from Turboflow columns using variable acetonitrile content

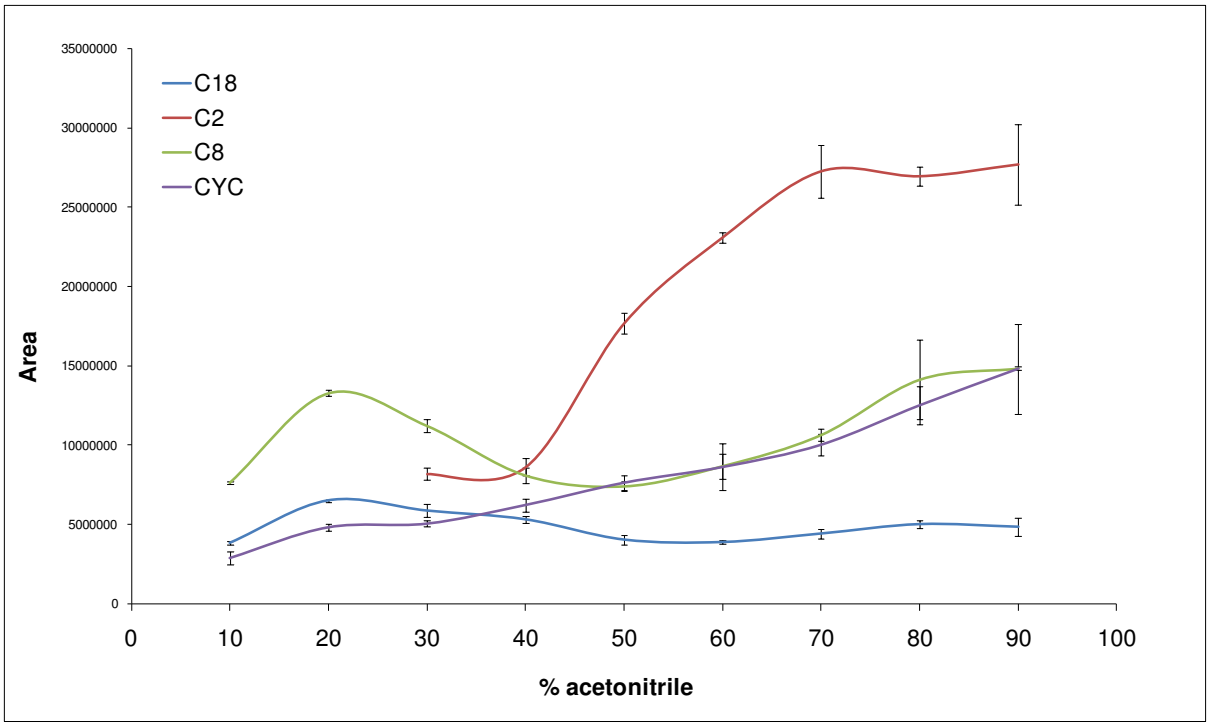


Figure 8-10; Recovery of phospholipid 787>184 from Turboflow columns using variable acetonitrile content

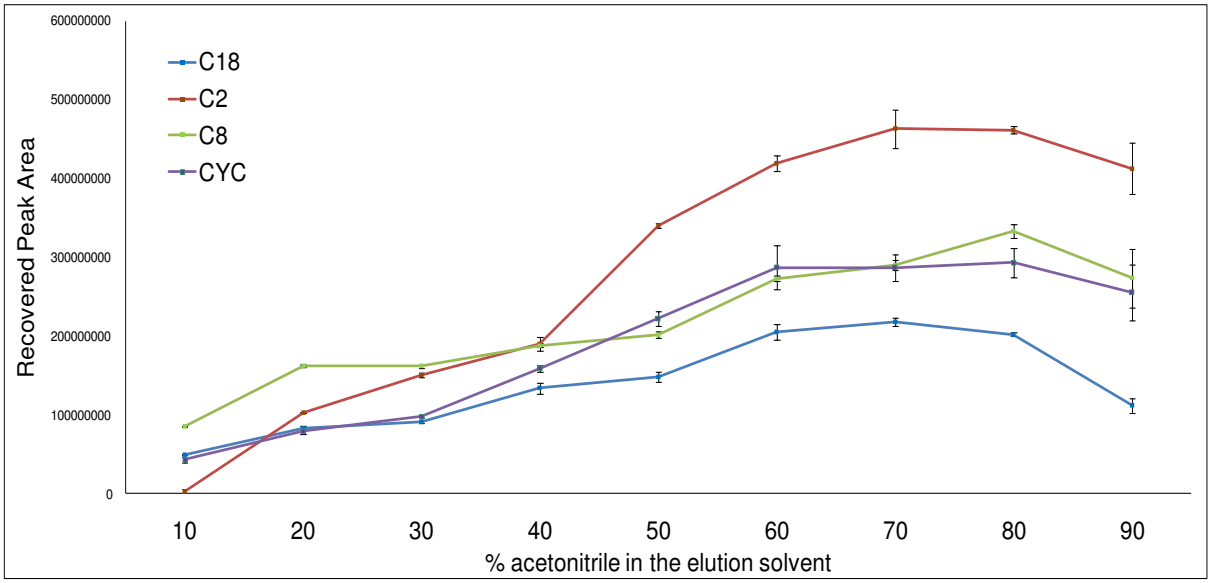


Figure 8-11; Recovery of total phospholipids showing peak area (±sd) from Turboflow columns using variable acetonitrile content

The data shows a variation in the profile of phospholipid recovery of each species on different columns with acetonitrile. Recovery generally increases proportionately with acetonitrile content of the elution mobile phase but was frequently found to reach a maxima where exceeding acetonitrile content reduced recovery. This may again be the result of a change in retention mechanism under a less polar environment. Overall, the C2

column was shown to recover a greater proportion of phospholipids than other phases with the exception of the species m/z = 496>184 Da which showed greater recovery and consequently more potential for contamination from the C18 column. Analysis of recovery data in 8.3.1 indicate that C8 columns gave the highest recovery of SARY at elution compositions greater than approximately 65% acetonitrile. The phospholipid recovery data was therefore reanalysed looking specifically at C8 column recovery with acetonitrile. The data presented in Figure 8-12 shows that while there is some proportionality with elution mobile phase organic composition, recovery of total phospholipid is consistent across the profile. This is reinforced by comparison of total phospholipid recoveries between columns in Figure 8-11 which show a relatively flatter profile across the range of elution gradient between 10 and 90%.

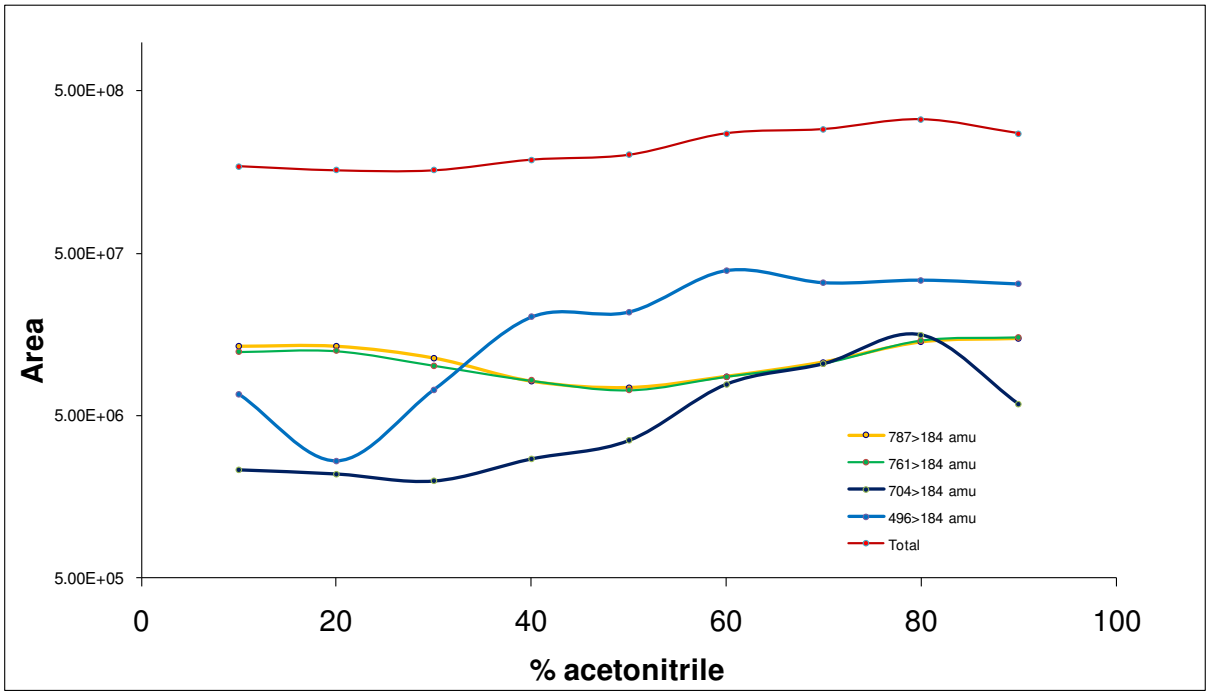


Figure 8-12; Recovery of individual probe and total phospholipids from C8 Turboflow columns using variable acetonitrile content

8.3.3 Elution Condition Selection

From the data obtained in 8.3.1 , C8 Turboflow extraction was selected as the optimum phase for the extraction and recovery of SARY. While C18 columns recovered less total phospholipid, recovery of SARY was less and required higher proportions of acetonitrile in the mobile phase limiting chromatographic retention which would be possible on a second analytical column. The actual recovery behaviour of SARY and total phospholipid from C8

column are compared in Figure 8-13 (phospholipid areas were reduced 1000-fold to enable representation on the same graph area). The comparison suggests that an acetonitrile composition greater than 70%, SARY recovery will be high while total phospholipid recovery will be moderate. This composition does not demonstrate either total recovery of SARY since this would require greater proportion of acetonitrile in the elution phase which would inhibit HPLC retention in the secondary column, neither does it demonstrate the absence of phospholipid recovery since by consideration of all other phospholipid recovery data shown in Figure 8-7 to Figure 8-11, it is not possible to recover zero phospholipids using extraction and mobile phase column combinations alone. The data presented indicates that the column and mobile phase selection gives an optimum condition of relatively high SARY recovery with relatively low phospholipid recovery from a single column switching extraction method.

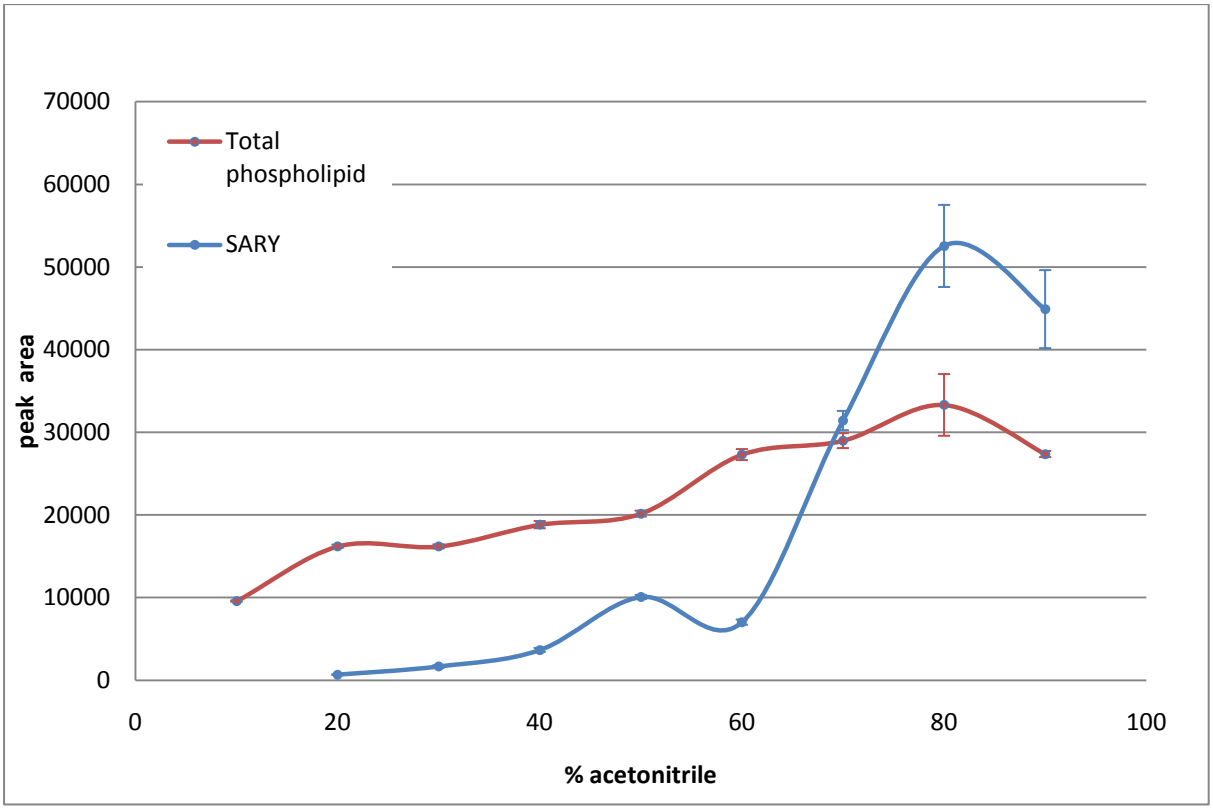


Figure 8-13; Recovery of SARY and total phospholipids (x 10-5) from C8 Turboflow columns using variable acetonitrile content mean of 3 determinations (±sd)

Examination of Figure 8-13 at low acetonitrile composition identifies the composition of elution mobile phase which may be exploited as a column phospholipid wash since at <20% acetonitrile, SARY is not detectable in elution from the extraction column while total

phospholipid recovery is approximately 20% of that at the intended elution composition. A <20% acetonitrile wash may therefore be applied to the Turboflow column after loading to further clean up phospholipids without removing extracted SARY. When SARY is subsequently eluted to the analytical column, the proportion of phospholipid eluted may be resolved from the peak chromatographically.

8.4 Chromatographic Separation

Chromatographic retention of SARY and 5 probe phospholipids was first assessed by eluting from the Turboflow column at 0.75 mL/min and chromatographing the eluent under isocratic conditions of the elution composition through a Gemini C18 50 x 3.0 mm i.d. analytical column (Phenomenex, Macclesfield). The retention times of the SARY peak and the probe phospholipids monitored in 8.3.2 and their peak areas (SARY x 10^5) are shown in Figure 8-14 and Figure 8-15. At 65% the difference in retention times between SARY and the probe phospholipids are sufficient to enable peak resolution of the target analyte from phospholipid interferences. This composition also corresponds to relatively low phospholipid recovery.

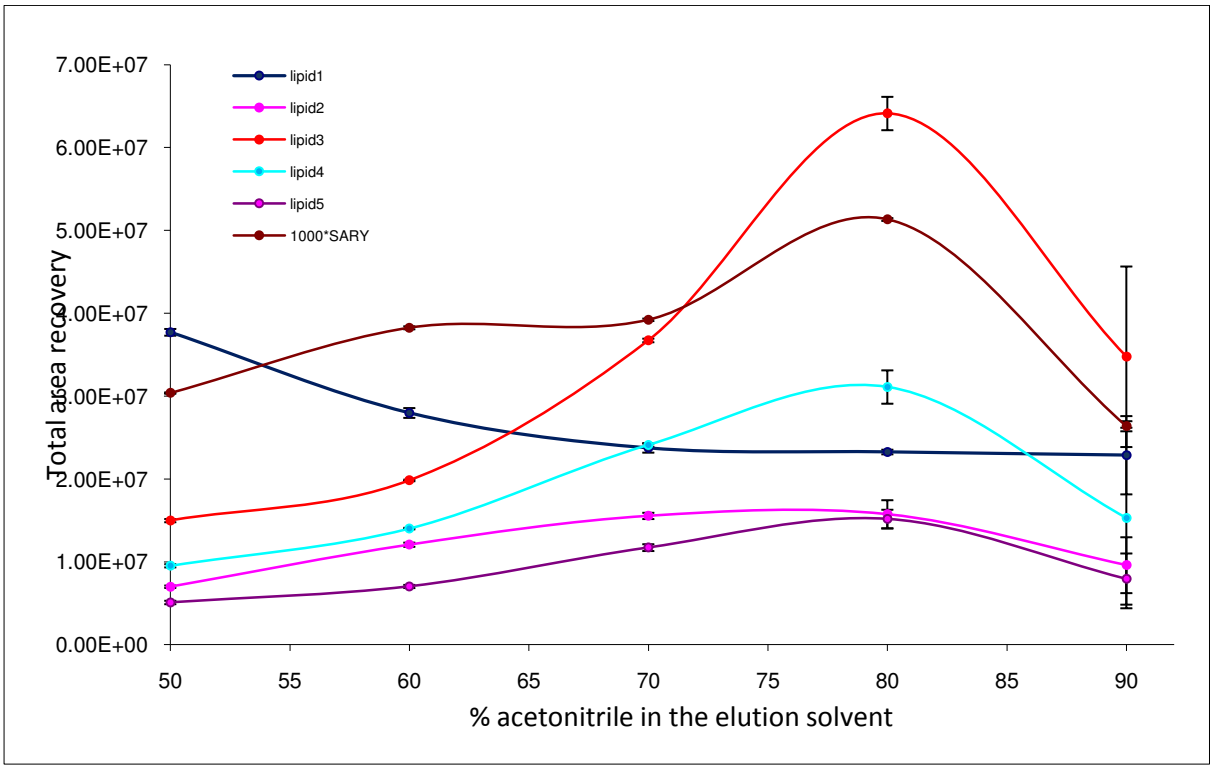


Figure 8-14; Plot of peak area SARY and 5 probe phospholipids eluted from a C8 Turboflow column to a C18 analytical column

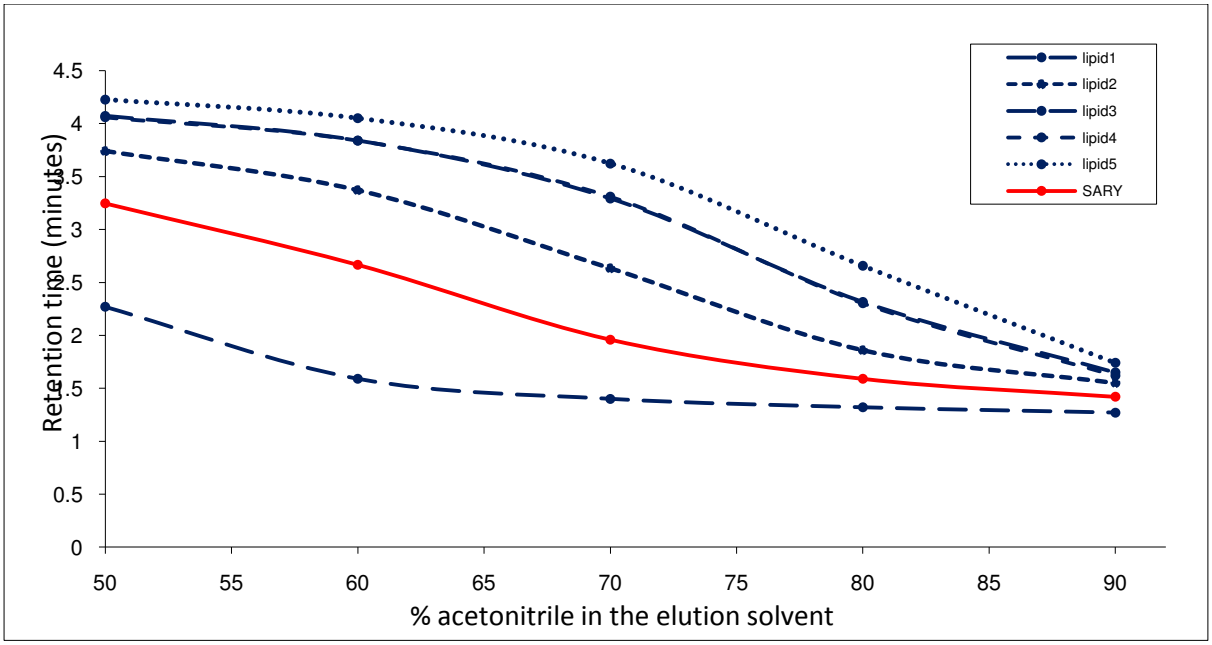


Figure 8-15; Plot of peak area SARY and 5 probe phospholipids eluted from a C8 Turboflow column to a C18 analytical column

Monitoring the total ion count of probe phospholipids, the mass chromatogram represented in Figure 8-16 demonstrates resolution of SARY from phospholipid interferences where in trace (a) at the selected transition for SARY, a peak is visible with a retention time of 3.38 and in the total ion count (TIC) trace of SARY and the probe phospholipids (b) there are no other peaks apparent at the SARY retention time. Furthermore, the mass chromatograms in Figure 8-17 obtained by injecting 50 µL of 6 batches of control human plasma which show (a) all precursors of m/z 184 and (b) full scan total ion count reveal that there is no detectable significant interferences at the same retention time as the drug peak.

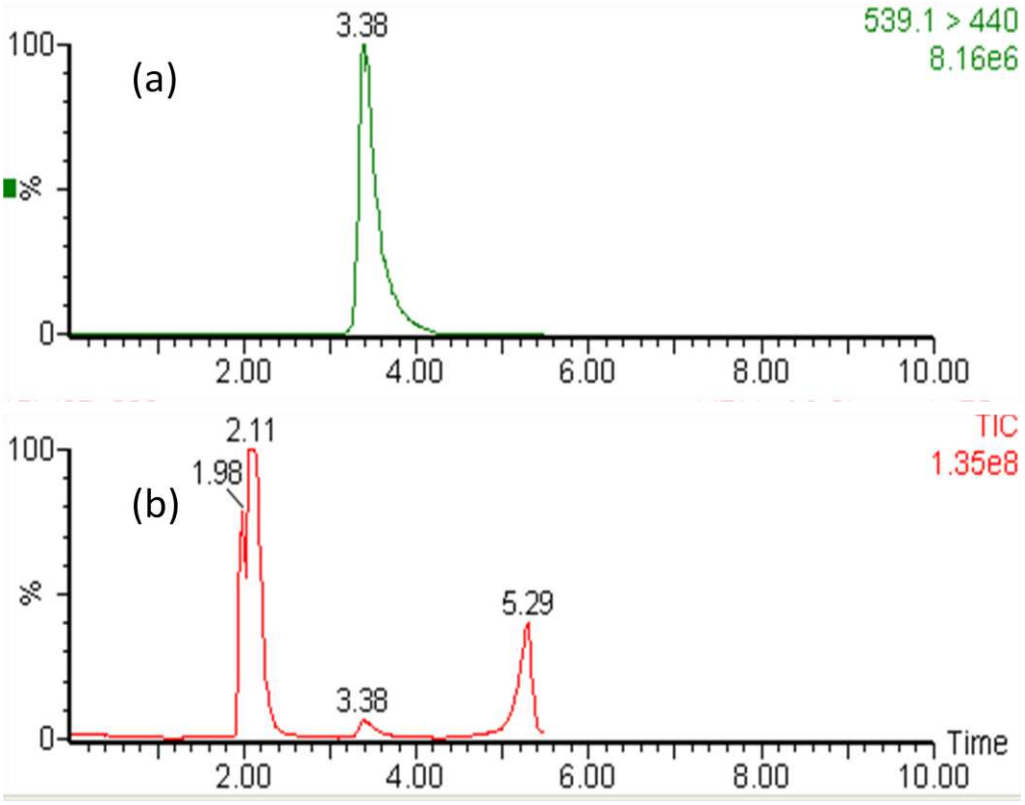


Figure 8-16; Mass chromatograms showing (a) selected transition for SARY from m/z 539.1>440 (b) total ion chromatogram of probe phospholipids and SARY

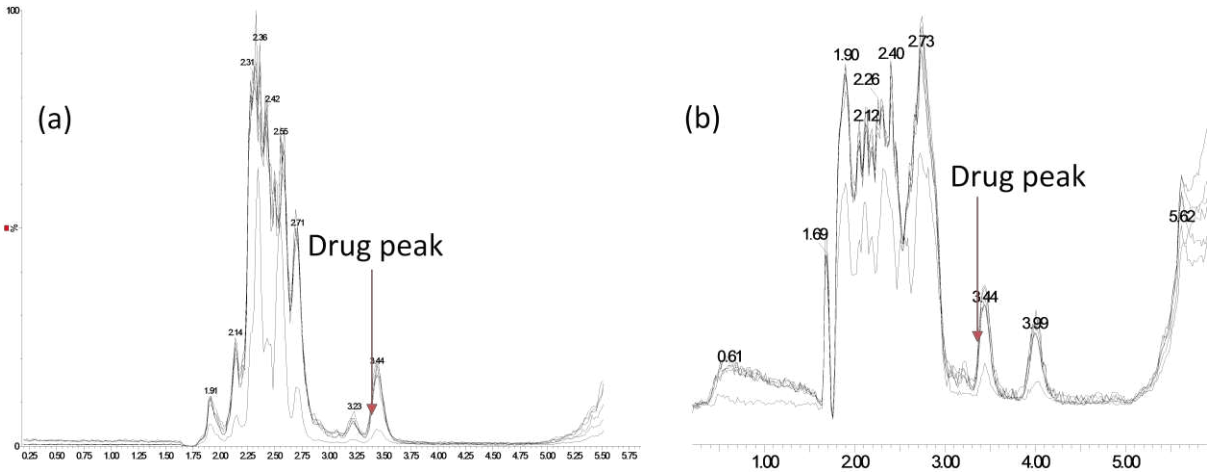


Figure 8-17; Sum of total ion chromatograms (n=6 plasma batches) monitoring (a) SARY and all precursors of m/z 184 Da and (b) Full scan mass chromatogram showing all detectable species.

8.5 Method Optimisation/Validation

Chromatographic procedure

The chromatographic procedure tabulated in Table 6-1 was constructed based upon the isocratic elution of SARY from a C8 Turboflow column with 65% acetonitrile and pH3.0 ammonium formate (20 mM). The use of a 0.5mm i.d. Turboflow column enabled elution of the analyte and transfer onto the analytical column in a smaller volume of mobile phase than used previously this was proven during method development to conserve peak shape as well as contributing to a reduction in solvent use.

Results and discussion

An LC-MS/MS mass chromatogram was generated from the selected reaction monitoring trace of 539.1 Da → 440 Da for SARY and 548.1 Da → 440 Da for [$^{2}H_{9}$]-SARY as shown in Figure 8-18 at the lower limit of quantification of the assay.

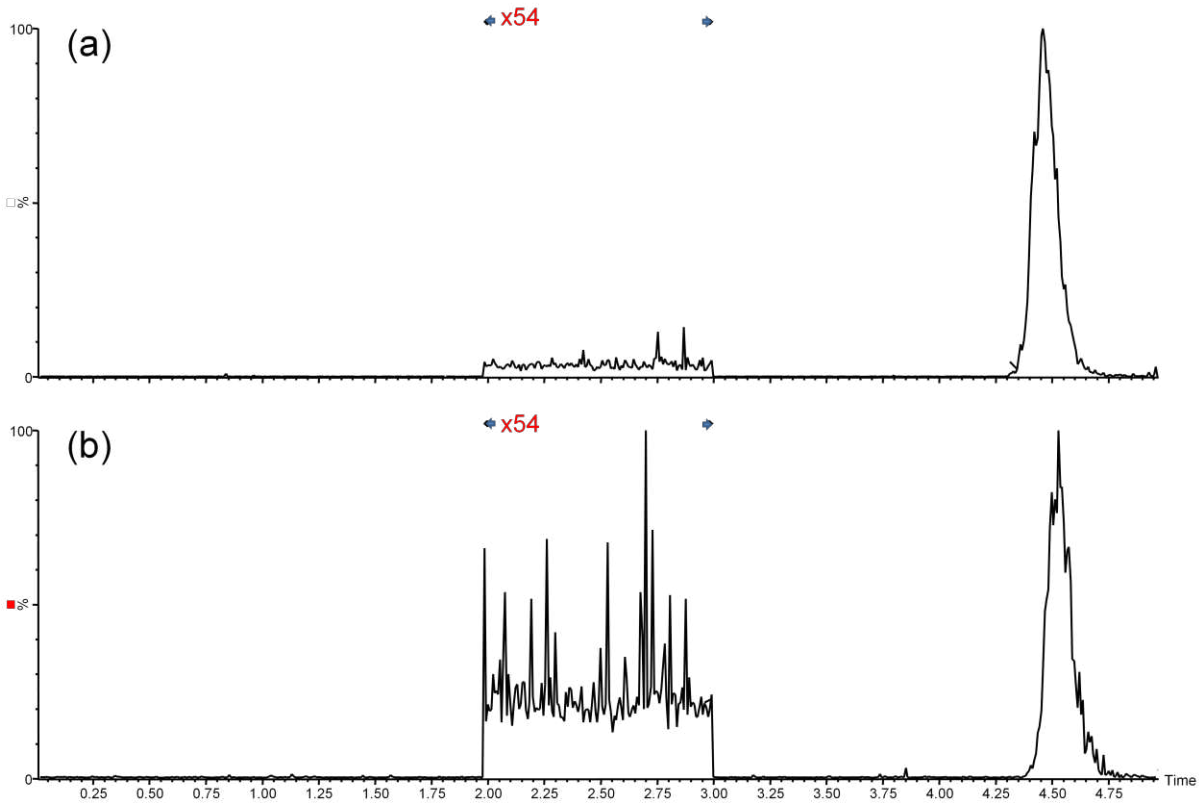


Figure 8-18; Representative product ion chromatogram of control human plasma spiked with SARY (20 ng/mL) and [$^{2}H_{9}$]-SARY (a) 548.1 Da → 440 Da (b) 539.1 Da → 440 Da

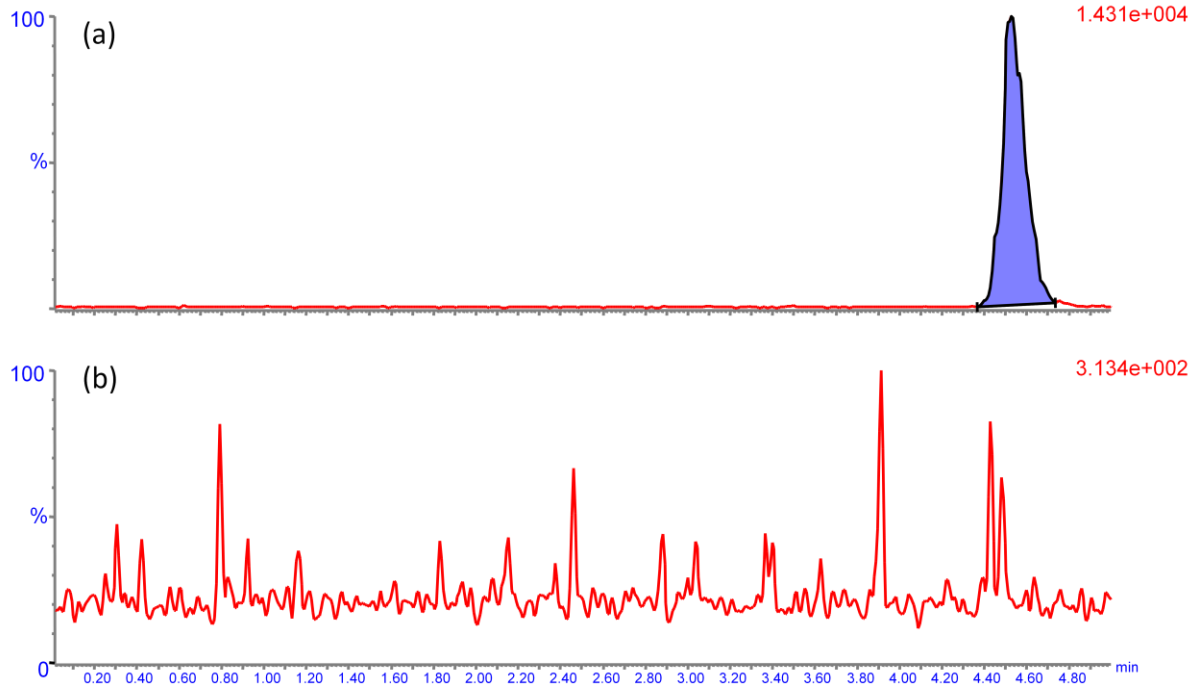


Figure 8-19; Representative SRM chromatogram of control human plasma spiked with SARY (20 ng/mL) (a) 539.1 Da → 440 Da (b) 548.1 Da → 440 Da

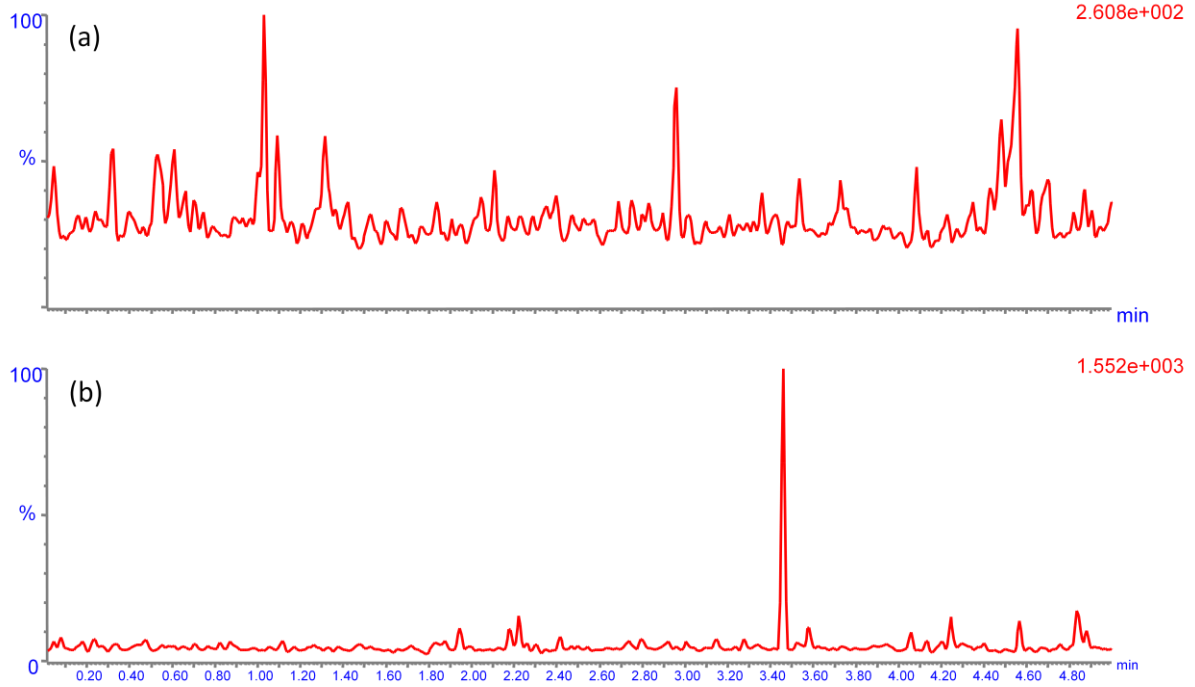
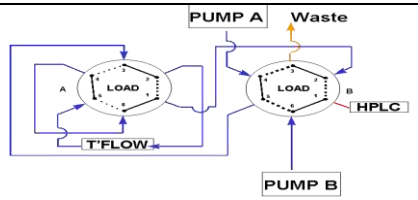
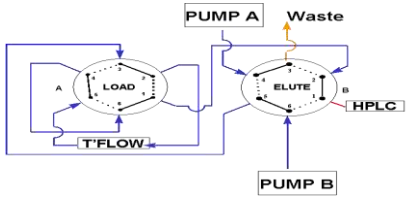
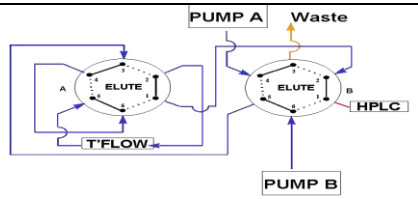
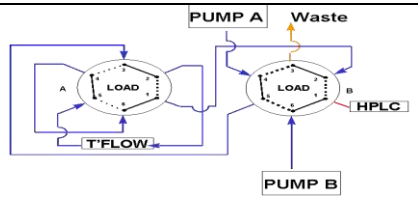


Figure 8-20; Representative SRM chromatogram of control human plasma (a) 539.1 Da → 440 Da (b) 548.1 Da → 440 Da

	Loading Mobile Phase (Pump A)			Analytical Mobile Phase (Pump B)				Valve positions and flow path	Description
Time (min)	Flow (mL/min)	% B	Valve 1*	Flow (mL/min)	% B		Valve 2*		
0.00	1.25	0	LOAD	0.75	65	Step	LOAD		Sample is injected in to the flow of mobile phase from the loading pump (A) and pumped through the Turboflow column. SARY is retained within by the C8 sorbent and unretained macromolecules are flow unretained to waste.
0.75	1.25	0	LOAD	0.75	65	Step	ELUTE		The direction of flow from the loading pump through the Turboflow column is reversed by switching valve 1 while retaining aqueous 100% conditions, flushing proteins and particulates trapped on the head of the column to waste.
1.25	1.25	10	LOAD	0.75	65	Step	ELUTE		10% acetonitrile is introduced from the loading pump to wash lipid material from the Turboflow column without elution of SARY.
1.75	1.25	0	ELUTE	0.75	65	Step	ELUTE		The elution pump (B) containing 65% acetonitrile is brought in line with the Turboflow column which is in turn brought in line with the analytical column by switching valve 2. SARY is eluted and transferred from the head of the Turboflow column and begins chromatographic separation through the analytical column.
2.75	1.25	90	LOAD	0.75	65	Step	LOAD		HPLC column is isolated from the Turboflow column by switching valve 2 while SARY is chromatographed through the analytical columns and 90% acetonitrile is pumped through the Turboflow column to clean up retained molecules in preparation for the next injection.

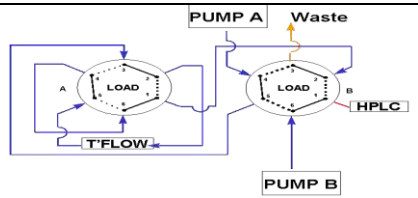
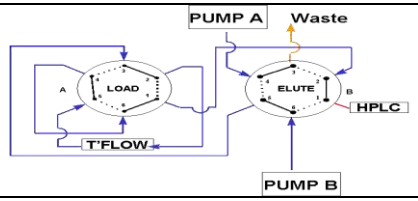
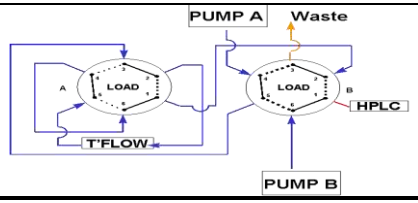
	Loading Mobile Phase (Pump A)			Analytical Mobile Phase (Pump B)				Valve positions and flow path	Description
4.75	**1.25**	**10**	**LOAD**	**0.75**	**90**	**Ramp**	**LOAD**		Following elution of SARY a gradient is applied to 90% acetonitrile from the elution pump (B) to elute any strongly retained components from the analytical column to prevent late elution during acquisition of the next chromatogram.
5.50	**1.25**	**90**	**LOAD**	**0.75**	**90**	**Step**	**ELUTE**		Valve 2 is switched to clean valve ports with 90% acetonitrile from the analytical pump.
6.50	**1.25**	**0**	**LOAD**	**0.75**	**65**	**Ramp**	**LOAD**		Valves are switched sequentially to clear valve rotor seals and flow paths. Mobile phase compositions return to starting conditions to clear organic solvent from the flow path of untreated plasma for the following injection to prevent protein denaturation.
6.75	**1.25**	**0**	**LOAD**	**0.75**	**90**	**Step**	**ELUTE**		
7.00	**1.25**	**0**	**ELUTE**	**0.75**	**90**	**Step**	**LOAD**		
7.17	**1.25**	**0**	**LOAD**	**0.75**	**65**	**Step**	**ELUTE**		
7.33	**1.25**	**0**	**LOAD**	**0.75**	**65**	**Step**	**LOAD**		Valve positions and mobile phase compositions returned to starting conditions to allow for re-equilibration.

Table 8-2; Chromatographic sequence

Calibration linearity, lower limit of quantification and absolute dynamic range.

The absolute sensitivity of the assay was determined to be at least 50 pg on column from plasma. This originates from a 10 µL injection of an aliquot of test sample (20 ng/mL) diluted 25% with internal standard in plasma. The resulting chromatogram produced a peak for SARY which gave a signal to noise significantly greater than 5-fold when compared to the amplified baseline. The expanded region of baseline in Figure 8-18 shows that the peak at the proposed lower limit of quantification is approximately 54 times baseline noise. In Figure 8-20 and it can be seen that blank control humans plasma did not contain detectable peaks with the same retention time as SARY in either the m/z = 539>440 Da transition or m/z = 548>440 Da indicating that there is no interfering signal arising from endogenous isobaric interferences, or in the m/z = 439>440 Da chromatogram in Figure 8-19 indicating that there is no contribution of isotopic cross-talk.

The calibration response was found to be linear between 20 ng/mL and 5000 ng/mL in plasma ($y = 0.00739x – 0.00491$; $R^2 = 0.997$). The inter-run and intra-run precision and bias was assessed at four concentrations across the calibration range by preparing 3 replicate groups of control samples and analysing them on 6 separate occasions (6 separate calibration lines; Table 8-3). The suitability of the assay for the determination of test samples was determined since each measure of precision and bias were within regulatory limitations of ± 15% (± 20% at lower limit) of expected concentration. [98] Inter-run precision was determined by calculating the coefficient of variation of the mean concentration results at each level from each of 6 runs, the intra-run precision was determined by calculating the mean of the coefficients of variation calculated for each concentration level in each of the 6 runs. The total precision of the assay was calculated from the coefficient of variation of all 36 results from each concentration level.

The impact of plasma matrix batch was also determined by analysing samples in plasma obtained from six human volunteers (three of either gender). Samples of plasma from each donor were analysed in replicates of 6. Acceptable precision and bias data within ± 15% nominal for each batch showed that the assay could be successfully applied without significant bias from individual variability (Table 8-4). A non paired t-test was calculated using the mean concentration result calculated from each donor to compare concentrations

detected in plasma from male and female volunteers. A calculated P value of 0.417 indicated that the difference between groups was not significant so no bias is associated with gender of the donors.

Nominal Concentration (ng/mL)	Plasma Concentration (ng/mL)			
	20	50	500	1000
Mean	17.0	45.4	493	4980
Bias (M%D) (95% Confidence Interval)	-15.1 (-19.5, -10.8)	-9.20 (-12.3, -6.12)	-1.41 (-4.25, 1.43)	-0.500 (-2.55, 1.55)
intra-run Precision (%CV) (95% Confidence Interval)	5.04 (3.61, 8.32)	5.95 (4.27, 9.82)	3.45 (2.48, 5.70)	4.66 (3.35, 7.70)
inter-run Precision (%CV) (95% Confidence Interval)	3.87 (0.00, 11.5)	3.45 (2.48, 5.70)	1.89 (0.00, 6.42)	0.00 (*,*)
Total Precision (%CV) (95% Confidence Interval)	6.36 (4.89, 12.7)	4.66 (3.35, 7.70)	3.94 (3.08, 7.47)	4.66 (3.35, 7.70)

Table 8-3; Inter- and intra-run precision and bias for the assay in human plasma

Matrix Variability	Plasma Concentration (ng/mL)						
	20 ng/mL						
Plasma Lot Number	Male 1 (n=6)	Male 2 (n=6)	Male 3 (n=6)	Female 1 (n=6)	Female 2 (n=6)	Female 3 (n=6)	P value (male-v-female)
Mean Precision (%CV) Bias (M%D)	17.5 2.88 87.5	18.2 8.41 91.0	18.3 5.79 91.5	18.2 4.74 91.0	17.1 4.46 85.5	17.6 6.82 88.0	0.417

Table 8-4; The impact of subject variability and gender on assay precision and bias

Biological and chemical stability

Pharmaceutical and exogenous molecules contained in blood plasma may undergo concentration changes as a result of both chemical and biological factors. [193] Therefore, chemical stability of the drug was evaluated in DMSO preparation solvent for up to 12 weeks and biological stability was evaluated in the plasma matrix for the same period of time under frozen conditions.

The absence of degradation or *ex-vivo* metabolism under the test conditions to greater than 15% deviation from expected concentration indicates that the assay is suitable for use following storage under the test conditions for the period tested (Figure 8-21). Additional stability studies were conducted to assess the stability of SARY in processed samples at room temperature for up to 7 days and in control human plasma when stored incubated at

37°C for 24 h to replicate approximate physiological conditions. The potential of instability during repeated freeze thaw cycles due to cryoprecipitation of bound proteins [79] was assessed by thawing the samples completely for at least 1 hour followed by frozen storage for at least 12 hours through 3 cycles. Under all stability conditions tested, the resulting concentrations of SARY remaining demonstrated a bias of less than 15% verifying that the integrity of the samples was unaffected by the storage condition.

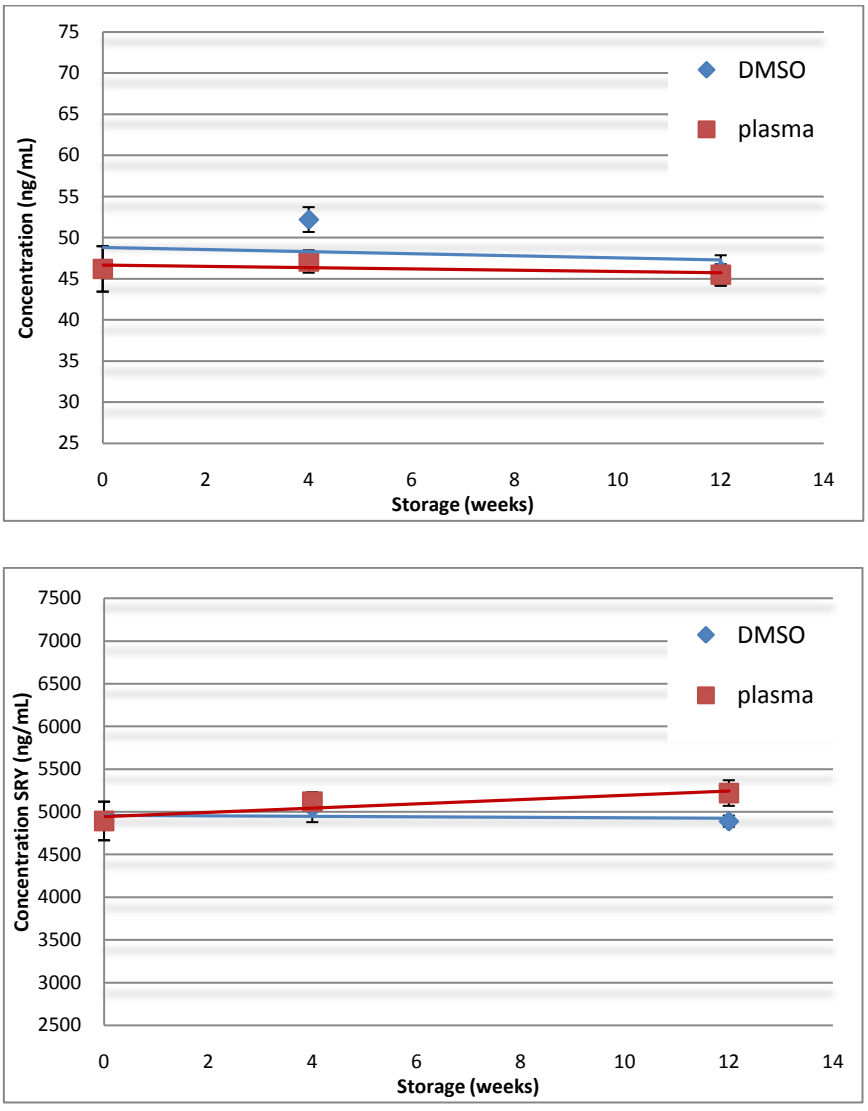


Figure 8-21; Stability of SARY in i) DMSO under ambient and ii) human plasma under frozen conditions

Stability in whole blood

The stability in SARY in whole blood was assessed by adding an aliquot of SARY in methanol (50:50 v/v) to whole blood at a ratio of 100 µL to 100 mL of whole blood to prepare blood samples of concentration 50 and 1000 ng/mL. The solutions were added at a high ratio of

blood to solvent volume to prevent excess haemolysis caused by the organic solvent. Immediately following preparation (time 0 hours) and then following 3, 6 and 24 hours under ambient conditions in upright tubes, the blood was centrifuged at 2500 rpm (1800 rcf) for 10 minutes to yield plasma which was immediately frozen and later analysed within the same run using the method described previously. The results of the analysis shown in reveal that SARY is stable (degradation/metabolism does not induce bias greater than 15% from value at 0 hours) for at least 24h. The plasma concentrations analysed are greater than blood concentrations due to the plasma volume proportionality to the overall blood volume. The actual ratio is a function of blood hematocrit and blood cell partitioning of the analyte.

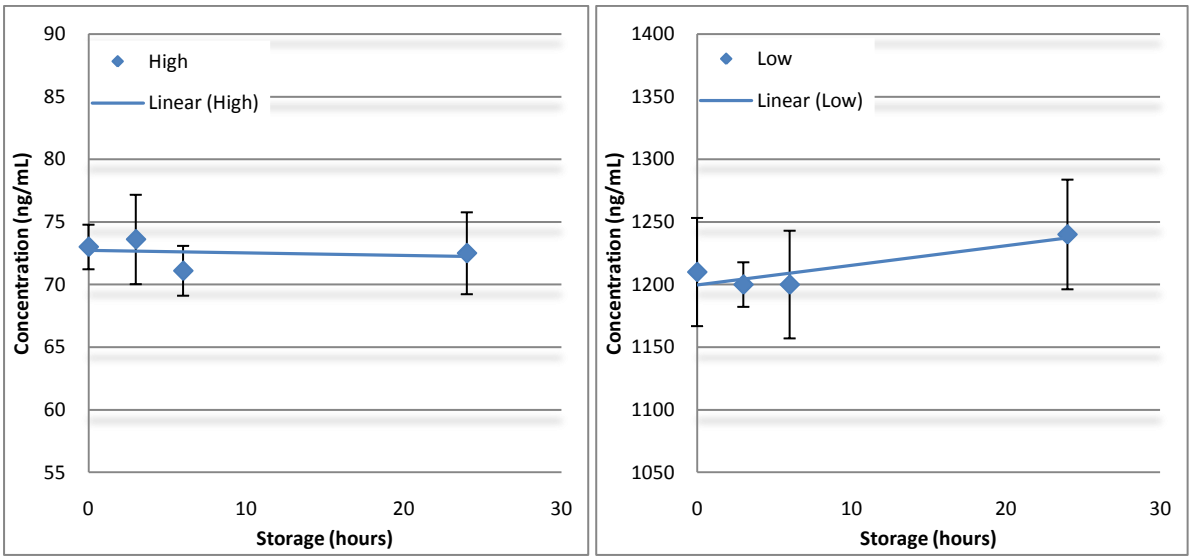


Figure 8-22; Stability of SARY in whole blood under ambient conditions

Factors influencing specificity of the assay

Factors with the potential to disrupt the assay as discussed in 6.2 were assessed to determine their impact on the determination of SARY. Inter-subject matrix variability, blood haemolysis and anticoagulant used were determined to have no significant effect on the assay with M%D and %CV of less than 15%. A number of concomitant medications and xenobiotic compounds anticipated to be present in test samples (paracetomol, asprin, ibuprofen, caffeine, levonorgestrel, ethylestradiol, ketoconazole, digoxin, warfarin, erythromycin, midazolam and simvastatin) were added to plasma at therapeutic concentrations used to prepare test samples of SARY which was analysed to demonstrate

specificity. In each case, precision and bias remained within acceptable limits of within 15% coefficient of variation or mean percent difference.

8.6 Equivalence of Human Ethnic Group and Dietary State

Since a difference was observed between the phospholipid distribution in lipemic plasma samples and those from fasted human volunteers described in chapter 7, a fixed ratio test described in chapter 6 was conducted on plasma samples that were visibly lipemic and those taken from volunteers who had fasted overnight. The two groups of samples were prepared at the lower limit of quantification in replicates of 18 samples from individual male volunteers of African American or Caucasian origin. The test was performed using just 500 µL of plasma from each individual sample by spiking 5 µL of a solution in DMSO containing 2 µg/mL SARY and 20 µg/mL [$^{2}H_{9}$]-SARY. The peak area ratio results were compared and analysed using an unpaired t-test. The data presented in Table 8-5 demonstrates that while phospholipid distribution has been shown to be separable between fasted and lipemic volunteers, the fixed area ratio test has shown quantitative equivalence between both groups since the calculated two tailed p-value of 0.189 is greater than 0.05.

Nominal conc (ng/mL)	Container	SARY/[2H9]-SARY Ratio	CV(%)	N	Mean difference	95% CI of mean difference	2 tailed p value
20	FASTED	0.128	2.60	18	0.00200	-0.00103 to 0.00503	0.189
	LIPEMIC	0.126	4.27	18			

Table 8-5; Equivalence of fixed ratio spiked SARY and [2H9]-SARY on human plasma from fasted and lipemic volunteers

The test was repeated next comparing human ethnic groups. Fixed ratio samples were prepared in plasma samples from 12 male Caucasian donors from both lipemic and fasted groups equally and compared to samples prepared from plasma from 12 male African American or Hispanic volunteers both fed and fasted groups. The data tabulated in Table 8-6 and Table 8-7 show that in both cases, the entire 90% confidence interval lies within 15% of an equivalence ratio of 1.

Nominal conc (ng/mL)	Container	SARY/[$^2H_9$]-SARY Ratio	N	Mean difference	95% CI of mean difference	2 tailed p value
20	Caucasian	0.129	12	0.00192	-0.00185 to 0.00569	0.3030
	Hispanic	0.127	12			

Table 8-6; Equivalence of fixed ratio spiked SARY and [2H9]-SARY on human plasma from Caucasian and Hispanic volunteers

Nominal conc (ng/mL)	Container	SARY/[$^2H_9$]-SARY Ratio	N	Mean difference	95% CI of mean difference	2 tailed p value
20	Caucasian	0.129	12	0.00333	-0.00061 to 0.00728	0.0935
	African American	0.125	12			

Table 8-7; Equivalence of fixed ratio spiked SARY and [2H9]-SARY on human plasma from Caucasian and African American volunteers

8.7 Validation of the Assay in Non Clinical Species

To support non clinical development studies, the assay was extensively tested using samples prepared in lithium heparinised control rat and dog plasma. The variability of the assay in plasma of each species was determined by analysing 4 concentration levels across the calibration range of the assay in replicates of 3 on 6 separate occasions. The results are summarised in Table 8-8 and Table 8-9.

Nominal Concentration (ng/mL)	Plasma Concentration (ng/mL)			
	20	50	500	5000
Mean	19.0	47.2	493	5740
Bias (M%D) (95% Confidence Interval)	-5.15 (-10.4, 0.101)	-5.26 (-9.76, -1.35)	-1.34 (-9.30, 6.61)	-5.26 (-14.2, 3.66)
intra-run Precision (%CV) (95% Confidence Interval)	5.00 (3.54, 8.49)	2.55 (1.83, 4.22)	3.98 (2.08, 10.3)	4.73 (3.34, 10.6)
inter-run Precision (%CV) (95% Confidence Interval)	4.37 (0.00, 12.3)	3.98 (2.08, 10.3)	7.29 (4.01, 18.7)	8.85 (5.29, 21.7)
Total Precision (%CV) (95% Confidence Interval)	6.64 (4.96, 13.4)	4.73 (3.34, 10.6)	8.41 (5.85, 19.2)	9.24 (5.93, 21.9)

Table 8-8; Inter- and intra-run precision and bias for the assay in dog plasma

Nominal Concentration (ng/mL)	Plasma Concentration (ng/mL)			
	20	**50**	**500**	**5000**
Mean	18.5	48.4	546	4700
Bias (M%D) **(95% Confidence Interval)**	-7.56 (-10.8, 4.29)	-3.12 (-5.46, -0.780)	9.13 (6.40, 11.9)	-6.00 (7.96, 4.04)
intra-run Precision (%CV) **(95% Confidence Interval)**	2.76 (0.936, 8.11)	2.12 (1.52, 3.50)	1.36 (0.978, 2.25)	1.57 (1.13, 2.59)
inter-run Precision (%CV) **(95% Confidence Interval)**	2.98 (0.936, 8.11)	1.95 (0.00, 5.51)	2.25 (1.21, 5.80)	2.63 (1.85, 5.97)
Total Precision (%CV) **(95% Confidence Interval)**	4.04 (2.99, 8.60)	2.88 (2.18, 5.95)	2.63 (1.85, 5.97)	2.36 (1.75, 5.05)

Table 8-9; Inter- and intra-run precision and bias for the assay in rat plasma

In both rat and dog plasma, stability was determined at -20 °C for up to 3 months, following at least 3 freeze/thaw cycles and in plasma for 24 hours under ambient conditions.

8.8 Surrogate Matrix Cross Validation

To assess the potential for cross validation between species, the fixed ratio test to verify the equivalence of ionisation efficiency between plasma sources was described in 6.7. This test was performed using analysis of samples by the assay constructed for SARY using rat, dog, guinea pig and monkey plasma and plasma from 2 different groups of human volunteers (Figure 8-23). To ensure inter-individual variability is incorporated, each sample (n = 9) was taken from a different individual or separate batch of pooled plasma for guinea pig and rat samples. The individual plasma samples used to prepare the test samples were those analysed for phospholipid distribution in chapter 7 where inherent differences in phospholipid distribution were demonstrated. The data shows the mean peak area ratio of each sample group with error bars showing ± standard deviation. The red horizontal lines highlighting ± 15% of the mean value of all samples demonstrates that the ratio of ionisation efficiency of SARY and [$^{2}H_{9}$]-SARY are not affected by the presence of endogenous components from control plasma from different species to an extent with the potential to infer unacceptable bias to cross validation within regulatory limits.[98] Furthermore the red dashed lines lying ± 5% of the mean value indicate that the dataset is entirely within 5%. The fixed ratio has demonstrated that differential suppression of ionisation should not bias cross validation of the assay between any of the groups tested and that the objective to use

a human plasma derived calibration line to perform regression for the quantification of samples in rodent plasma is possible.

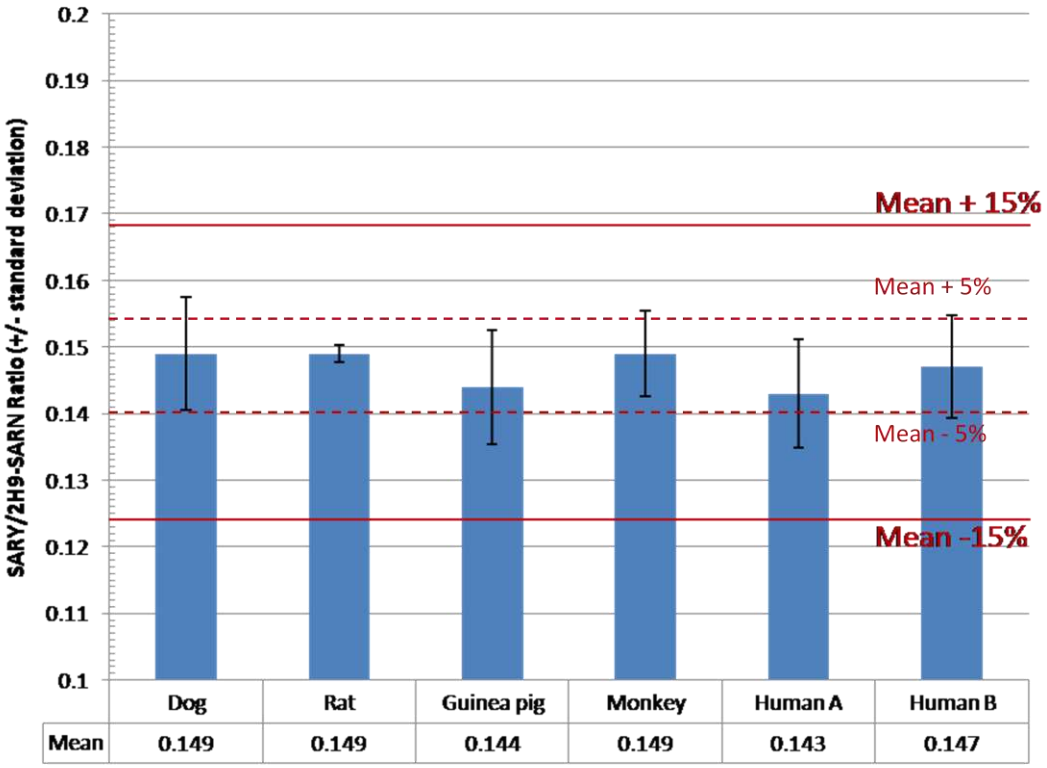


Figure 8-23; Fixed ratio ionisation equivalence test of SARY and [2H9]-SARY prepared in control plasma from several species

The ability to quantify samples in rodent plasma using a calibration line derived from plasma from a surrogate species was verified by repeating the assay variability investigation described in section 8.7 using control samples from across the calibration range prepared in mouse and rat plasma and analysing them using a calibration line prepared in human plasma. The analysis was carried out using 3 replicates at 4 concentrations and performed on 6 separate occasions (Table 8-10, Table 8-11). The test concluded that for both rat and mouse plasma samples, precision and bias were within accepted regulatory limits (± 15%; 20% at lower limit of quantification) required to support nonclinical pharmacokinetic studies. [98, 225]

Nominal Concentration (ng/mL)	Plasma Concentration (ng/mL)			
	20	50	500	1000
Mean	18.9	50.6	521	5360
Bias (M%D) (95% Confidence Interval)	-5.69 (-18.2, 6.79)	1.23 (-8.25, 10.7)	4.22 (-0.00241, 8.45)	7.21 (1.92, 12.5)
intra-run Precision (%CV) (95% Confidence Interval)	9.23 (6.62, 15.2)	8.64 (6.20, 14.3)	5.53 (3.97, 9.13)	3.05 (2.19, 5.04)
inter-run Precision (%CV) (95% Confidence Interval)	11.4 (4.83, 30.5)	7.40 (0.00, 21.3)	2.17 (0.00, 8.89)	4.36 (2.15, 11.4)
Total Precision (%CV) (95% Confidence Interval)	14.7 (10.7, 31.9)	11.4 (8.67, 23.2)	5.94 (4.68, 10.9)	5.32 (3.81, 11.8)

Table 8-10; Inter- and intra-run precision and bias for the assay in mouse plasma regressed using a calibration curve prepared using human plasma

Nominal Concentration (ng/mL)	Plasma Concentration (ng/mL)			
	20	50	500	5000
Mean	16.8	47.5	502	5080
Bias (M%D) (95% Confidence Interval)	-16.0 (-29.1, -2.90)	-4.97 (-8.30, -1.63)	0.456 (-8.04, 8.95)	1.60 (-2.59, 5.79)
intra-run Precision (%CV) (95% Confidence Interval)	15.2 (10.8, 25.8)	5.89 (4.22, 9.72)	12.6 (9.07, 20.9)	4.89 (3.46, 8.30)
inter-run Precision (%CV) (95% Confidence Interval)	11.8 (0.00, 34.3)	0.00 (*, *)	3.41 (0.00, 18.3)	2.65 (0.00, 8.88)
Total Precision (%CV) (95% Confidence Interval)	19.2 (14.5, 38.0)	5.89 (4.22, 9.72)	13.1 (10.3, 23.4)	5.56 (4.26, 10.4)

Table 8-11; Inter- and intra-run precision and bias for the assay in rat plasma regressed using a calibration curve prepared using human plasma

8.9 Assay Equivalence using Incurred Samples

The method was applied to the analysis of incurred samples which had been drawn to generate exposure data from a pharmacokinetic study in mice. The animals were dosed with two test intravenous formulations of SARY and samples taken at time intervals following dosage to calculate the effect of dosage formulation on pharmacokinetic exposure to the drug. Samples were initially analysed using the validated method of analysis described previously using an internal standard prepared in mouse plasma and quantified using a set of calibration samples prepared in mouse plasma. The data was reported to the drug development project team to support optimisation of the drug target formulation. In total, 38 samples were analysed with calculated concentrations ranging from 37.0 to 24200 ng/mL. Where concentrations were found to exceed the upper range of the calibration

curve, an aliquot was diluted 10-fold using control mouse plasma by adding 50 µL of mouse plasma sample to 450 µL of control mouse plasma. [225]

The samples were subsequently reanalysed using a calibration curve and working internal standard prepared in human plasma and a summary of each dataset data compared in Table 8-12, with extensive data recorded in Appendix 2. The two-tailed P value of 0.494 demonstrates that by conventional statistical criteria ($p>0.05$), there is no significant difference between the two sets of data, [226] since the probability that observed differences result from random error is greater than 5%. Therefore the determination of concentration of the test samples performed using a surrogate matrix of human plasma based calibration curve is equivalent to that obtained using matrix equivalent mouse plasma. The comparison presented in detail in Appendix 2 shows that since the overall percentage bias and coefficient of variation are within 20%, and individually, less than 33% of values differ from the control value (derived from mouse plasma calibration) by greater than 20% in compliance with regulatory requirements [227, 228] of incurred sample reproducibility, the assay was therefore presented as being equivalent in the analysis of *ex-vivo* samples in mouse plasma following linear regression using calibration samples prepared in human control plasma.

Group	Human	Mouse	Mean Difference	95% CI	Two-tailed P value
Mean	3303.811	3200.792	103.018	-199.049 to 405.086	0.4939
SD	6860.231	6087.550			
SEM	1112.876	987.531			
N	38	38			

Table 8-12; Incurred sample reproducibility using plasma samples analysed using calibration standards prepared in rat and human plasma

8.10 SARY Method Conclusions

An assay was developed and optimised for SARY in plasma from human and some non-clinical species and thoroughly assessed and demonstrated to be suitable for the analysis of specimens in support of non-clinical safety studies and clinical trials in compliance with criteria required by international regulatory authorities governing study conduct in the pharmaceutical industry. The assay employed on-line Turboflow column extraction from plasma macromolecules which enabled efficient inclusion of extraction and analysis steps in a single operation while the use of narrow bore Turboflow columns reduced the solvent

requirements due to reduced flow rate by comparison with standard bore columns used previously.

With consideration to the reduction of extraction and chromatographic resolution of endogenous glycerophosphocholines, a major source of matrix effect was controlled in the assay. A test using fixed ratio solutions of analyte and stable isotope labelled internal standard was applied and used to demonstrate that any residual matrix effect between plasma of different species did not induce quantitative bias when each was compared. The validity of use of calibration samples prepared in human plasma to analyse control samples in rodent plasma was determined in rat and mouse samples demonstrating that the assay could be used to quantify rodent plasma samples using human control matrix. The assay was finally applied to the analysis of study samples in mouse plasma using both matrix matched calibration samples and surrogate calibration samples prepared using human plasma. Statistical analysis of the data generated showed that there was no significant difference between the datasets generated using the species matched and the surrogate species plasma control matrix to quantify study samples, therefore the intention to optimise the assay to control inter-species matrix effects enabling the use of a surrogate control plasma was successful in achieving its objectives.

9 Dried Blood Spot Method for SARY

9.1 Introduction

To investigate the potential of the assay for SARY evaluated in chapter 8 to quantify samples extracted from dried blood spots of human and animal origin as described in chapter 4, extraction of SARY was investigated using a number of available paper matrices as listed in Table 3-1. The Turboflow extraction was initially retained to enable injection of the anticipated organic extract into a highly aqueous mobile phase in order to retain the analyte on the extraction column for transfer to the analytical chromatography column with maximum control over chromatographic migration.

9.2 Preparation and Spotting of Whole Blood Samples

Samples were prepared in whole blood by adding 50 µL of SARY in DMSO to 4.95 mL of human or rat whole blood which was either freshly drawn or stored under refrigerated conditions. After addition of SARY solution, the samples were very quickly vortex mixed to minimise haemolysis and rotary mixed for at least 10 minutes. 100 µL spots were spotted directly to each test card and allowed to dry at room temperature in a biological safety cabinet for at least 3 hours. Following surface drying, the cards were transferred and stored in a desiccator to ensure controlled completion of the drying process for at least 1 week. Upon spotting of test samples, visible differences in the formation of blood spots were observed as indicated in Figure 9-1. The 'halo' effect of diffusion of the blood serum content beyond the bulk blood cell fraction is observed with FTA elute® paper (Table 3.1) while rapid and complete red blood cell lysis; breakdown of the cell membrane to release the cell contents, gave rise to the rapid change in colour to dark brown/black from red. To prevent bias caused by distribution effects which might be associated with these observations, samples were cut from as close to the centre of the blood spot as possible. Since blood samples may be spotted at body temperature or following refrigerated storage, the effect of blood temperature on the rate of diffusion of the spot into the paper was investigated by spotting different paper types with 100 µL of blood (to obtain measurable differences) from aliquots of whole blood pooled from the same donor stored for 1 hour under refrigeration, at ambient temperature, or following 1 hour incubation at 37°C. Spots were added at controlled rate by using an electronic pipette (Rainin EDP 100)

and the time measured between completion of the dispense step to the disappearance of the final drop of pooled blood below the pipette tip on the paper surface. Following drying, the spots formed were measured by ruler as tabulated in Table 9-1. The effect of temperature on blood viscosity was shown to affect the time taken for blood diffusion into the paper matrix; however, this did not affect the size of the spot formed.

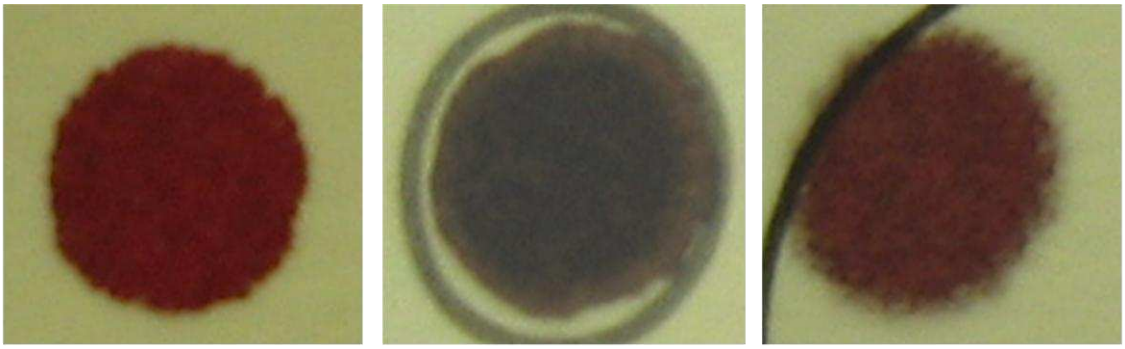


Figure 9-1; Appearance of fresh human whole blood (30 µL) spotted onto DBS paper media (1) IDB 226, (2) FTA elute, (3) FTA

An additional factor which may affect distribution of blood on paper is temperature since samples are drawn at body temperature while drug stability considerations may require rapid chilling of blood samples. As blood cools, changes in viscosity may change the rate of distribution through the paper and consequently the size of spots formed per unit volume. Any temperature effect on the size of spots formed may induce sampling temperature dependant bias on the analytical process. To assess this, a pool of freshly drawn human whole blood in potassium EDTA was aliquotted into identical storage tubes which were stored for 10 minutes in an incubator set to 37°C, at room temperature, and on ice (0°C). Following thermal equilibration, the blood samples were spotted onto IDB226 paper (25 µL; n = 6) using a rate controlled digital pipette. Following an audible signal at the completion of the pipette dispense function; the time was measured until the pool of wet blood formed below the tip was observed to diffuse into the paper. After complete drying and desiccation, the diameters of the spots formed were measured. The results tabulated in Table 9-1 indicate that while the temperature of blood on spotting effects the rate of distribution through the paper, this has no effect on the diameter of spots formed.

	Replicate	1	2	3	4	5	6
Blood equilibration temperature	*Time to spot distribution (seconds)*						
	37°C	3	3	3	3	3	3
	Room Temp	5	5	4	4	4	4
	0°C	5	6	6	5	6	5
	Spot diameter (mm)						
	37°C	16	16	15.5	16	16	16
	Room Temp	16	16	16	16	16	16
	0°C	16	16	16	16	16	16

Table 9-1; The effect of blood sample temperature on the speed of percolation and size of blood spots in paper

9.3 SARY Recovery from DBS Paper

Initial comparison of the recovery of SARY from paper revealed that FTA® paper provided the highest recovery of SARY from the paper matrix when compared with FTA elute® and untreated Ahlstrom 226 paper. A simple stepwise method for the recovery of SARY from FTA® paper was therefore optimised as follows;

- A 5.0 mm i.d. spot was cut from the FTA card and transferred to a 2.0 mL Eppendorf tube.
- Internal standard solution (200 µL of [$^{2}H_{9}$]-SARY; 5 ng/mL in methanol) was added to the tube and vortex mixed thoroughly for approximately 10 seconds then allowed to stand for at least 2 minutes to extract the punched sample.
- A further 100 µL of pure water was added to the tube to correct the solvent composition and vortex mixed.
- The entire recoverable volume of extraction solvent (approx 300 µL) was transferred into a 96-well plate by pipette, taking care not to pick up solid filter paper.
- The plate was sealed with a square well pierceable sealing cap and loaded into a deep-well stack of HTS PAL auto sampler before injection of 100 µL of extract using the method described in chapter 8.

A typical chromatogram obtained by this method is presented in Figure 9-2 showing similar retention characteristics to the method described in chapter 8 and a clearly greater than 5-fold signal to noise ratio satisfactory for the lower limit of quantification.

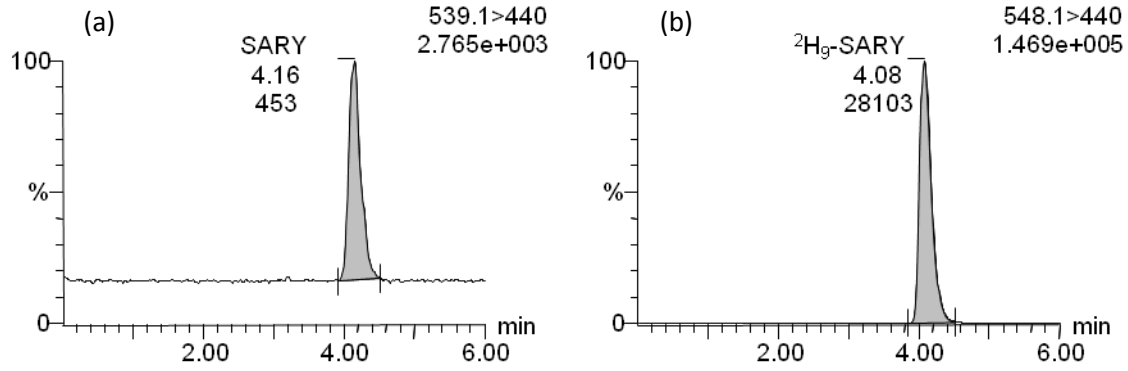


Figure 9-2; Chromatograms of (a) SARY and (b) [2H9]-SARY extracted from FTA paper dried spots of human blood

Influence of vortex mixing time on extraction recovery

To evaluate the effect of time vortex mixing the punched blood spot in methanol has on the recovery of extraction of SARY from dried blood on the paper matrix, 4.2 mm punches (n=4) were sampled from human blood spotted on FTA paper, and vortex mixed in 200 µL of methanol for 10, 20 and 40 seconds before dilution with 100 µL water. The sample was vortex mixed for a further 10 seconds then the liquid was removed to a microtitre plate for injection. Additionally, to assess the effect of standing without vortex mixing, punches were mixed with methanol and vortex mixed for 10 seconds followed by standing for a further 50 seconds, then mixing with water and removal of the liquid.

The results in Figure 9-3 show that extraction recovery increased with time from 10 to 40 seconds, however, an increase was also observed following a short vortex mix followed by standing in the extraction solvent, greater than the recovery attributed to 10 seconds mixing alone but not as much as vortex mixing for the entire period since vortex mixing for 40 seconds achieved greater recovery. This indicates that following mixing, the compound continued to release from the paper into the extraction solvent although at a slower rate. For practical reasons, it was decided to mix samples for 10 seconds followed by standing for at least 1 minute for later experiments.

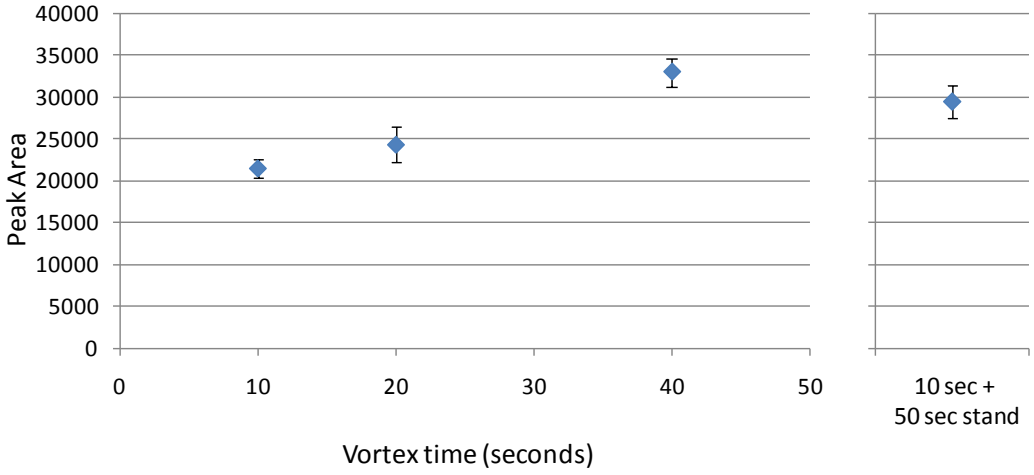


Figure 9-3; The effect of vortex mixing time in the extraction solvent on the recovery of SARY from dried blood spots

Preparation of human whole blood analytical samples

Blood calibration samples were prepared by mixing control human whole blood (4.95 mL) with an aliquot (50 µL) of SARY standard solution in DMSO to give concentrations of 20, 50, 100, 250, 500, 1000, 2500, 5000 ng/mL. A second set of validation test standard samples were prepared in control human whole blood at 20, 50, 500, 5000 ng/mL. Blood samples were spotted onto FTA® paper and allowed to dry at room temperature for 3 hours then transferred to a desiccator for at least 1 week prior to analysis. A working internal standard (5 ng/mL) was prepared in methanol.

The extraction method was evaluated by analysing a full set of calibration samples together with 6 replicates of the validation samples across the calibration range in a run bulked out to 96 samples with blank samples prepared from the working internal standard. The replicates of quality control samples across the calibration range were to assess the precision and bias of the assay which were required to demonstrate compliance with acceptable limits of precision and bias (15%; 20% at LLOQ) prescribed by the regulatory authorities for the conduct of analysis of samples for drug development studies. [98] Extending the run size to 96 samples with the inclusion of groups of blank samples prepared by addition of aliquots of the reconstitution solvent (300 µL of methanol/water; 2:1) was intended to determine that the robustness of the assay across the maximum intended run size of 96 samples did not compromise precision and bias of the assay. The results of the

analysis summarised in Table 9-2 show that the method was acceptable for use resulting in precision less that 5% coefficient of variation consistently at all levels while bias was less that 4% with the exception of the upper level where it was 10%.

Sample Name	Nominal Sample Concentration (ng/mL)	Mean Detected Concentration (ng/mL).	Bias (M%D)	Precision (%CV)
Val LLOQ	20	20.6	2.75	4.54
Val LOW	50	48.9	-2.27	3.94
Val MID	500	481	-3.87	3.75
Val HIGH	5000	5510	10.1	3.25

Table 9-2; SARY dried blood spot validation results

9.4 Method Optimisation/Validation

To further optimise the reconstitution of SARY extracted from dried blood spots, a multivariate analysis screening experiment was designed using the variables identified during preliminary method development as important in influencing recovery. The extraction process was divided into 2 stages, firstly pH adjustment or aqueous control of the blood spot on paper followed by controlled mixing and equilibration then extraction into an HPLC compatible solvent (methanol, acetonitrile or water). Whilst it is known that the log D values of SARY are constant between pH 2 and pH 10 therefore pH adjustment within this range is unlikely to influence analyte ionisation, changes in the pH environment, ionisation or polarity of the cellulose paper matrix or associated chemical additives may influence the elution of associated SARY from the paper. Whole blood samples were prepared at 5000 ng/mL in freshly drawn human blood and as shown in Figure 9-4, 25 µL aliquots were spotted onto sample papers of 2 types (FTA® and IDB226) and dried at room temperature for at least 3 hours followed by storage in a desiccator for at least 1 week. Before analysis, a 4.2 mm sample was punched into each cell of a 96 well microtitre plate using a BSD600 robot then test extraction solutions applied. The experimental design for the screening experiment followed a factorial design calculated using MODDE9 (Umetrics, Umea, Sweden). Adjustment of pH was done by the addition of 125 µL of either 1 M formic acid or 1 M ammonium hydroxide while the contribution of pre-wetting with no pH adjustment was tested by the addition of pure water. Following addition of wetting solvent, the samples

were mixed for 10 minutes on a MicroMix 5 eliptical shaker (Siemens diagnostics, Deerfield, IL, USA). In the second step, extraction solvent was added (methanol, acetonitrile or pure water) as 125 µL aliquots or mixtures prompted by MODDE factorial design totalling 125 µL. Where no solvent was added in the first step, 125 µL of extraction solvent was added in step 2 to ensure approximately equal surface exposure of the blood spot disk to the extraction solvent. After addition of the extraction solvent, the plate containing test samples was shaken for a further 10 minutes and left to stand for 1 hour to complete the extraction. The entire supernatant volume was withdrawn by pipette to a second microtitre plate and a 50 µL aliquot was analysed for SARY using the method described in chapter 8.

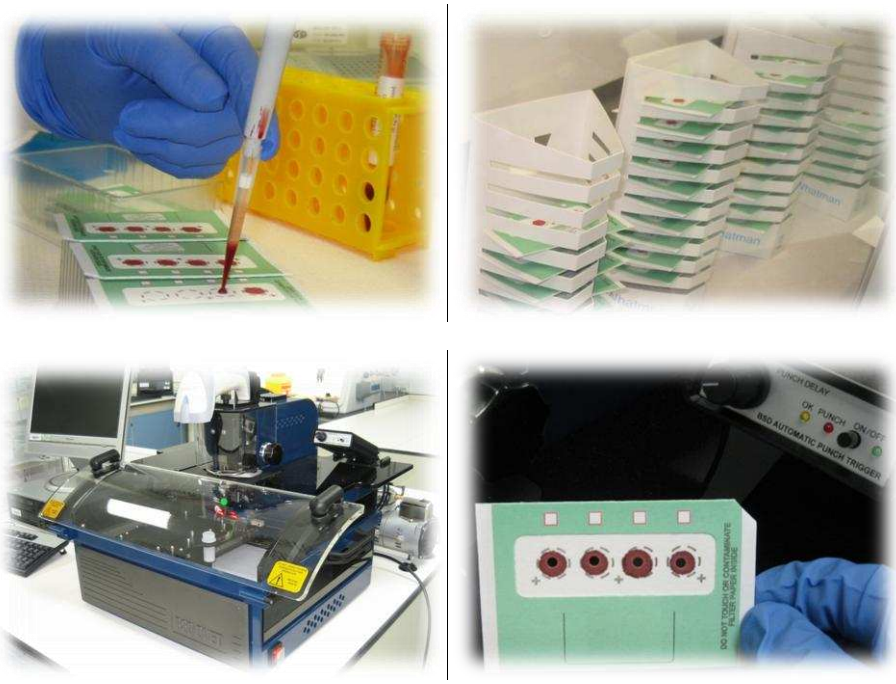


Figure 9-4; Spotting whole blood, drying in racks and punching using a BSD600 robotic punch

The difference between extraction conditions were visually evident as shown in Figure 9-5, the significance of each condition investigated in the experiments are described by the scaled and centred coefficients plot in Figure 9-6. This is produced by plotting the peak area data resulting from the contribution of each test condition and centring the data on an average value, resulting in a graphical representation of the contribution of each component since centring reveals a positive or negative influence on recovery. The plots are scaled showing residual values of peak area rather than percentage normalised which is useful in this instance to compare absolute values between paper types. It is apparent that for each paper type tested, recovery is smallest with acidification of the sample and greatest

when the pH is increased. Extraction with acetonitrile provides the best recovery demonstrated by a positive bar on each chart while water provides poor recovery shown by a negative bar. It appears from the coefficients plot that extraction in methanol results in a negative value; however, this is a relative result of the centralisation of the coefficients plot and reflects the magnitude of the acetonitrile result. Bars to the right of each plot show summed results of both stages in the extraction process. The first mixed value in both charts show that adjustment using formic acid increases the recovery of methanol extraction; however, it is interpreted from the left part of each chart that methanol extraction is less favourable than acetonitrile extraction. The combination of ammonium hydroxide adjustment with acetonitrile extraction produces a slightly positive trend.

The contours plot in Figure 9-5 shows more clearly the interaction of each component. For each paper tested, three triangular plots are produced showing the proportion of each of the 3 extraction solvents across each axis. For the conditions in stage 1, adjusting with formic acid, ammonium hydroxide, or no adjustment, a graph is populated showing colour scaled calculated recovery data with the scale shown to the right of each plot. It is clear for each plot from each paper type that greatest values are observed from acetonitrile extraction with highest recovery following pH increase. The magnitude of peak areas recovered under optimum conditions confirms that recovery is greatest from FTA® paper.

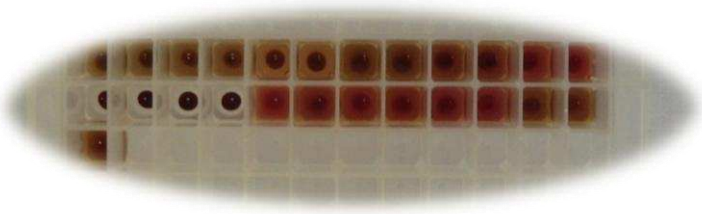


Figure 9-5; IDB226 paper punched and extracted in test solvents using the MODDE screening method

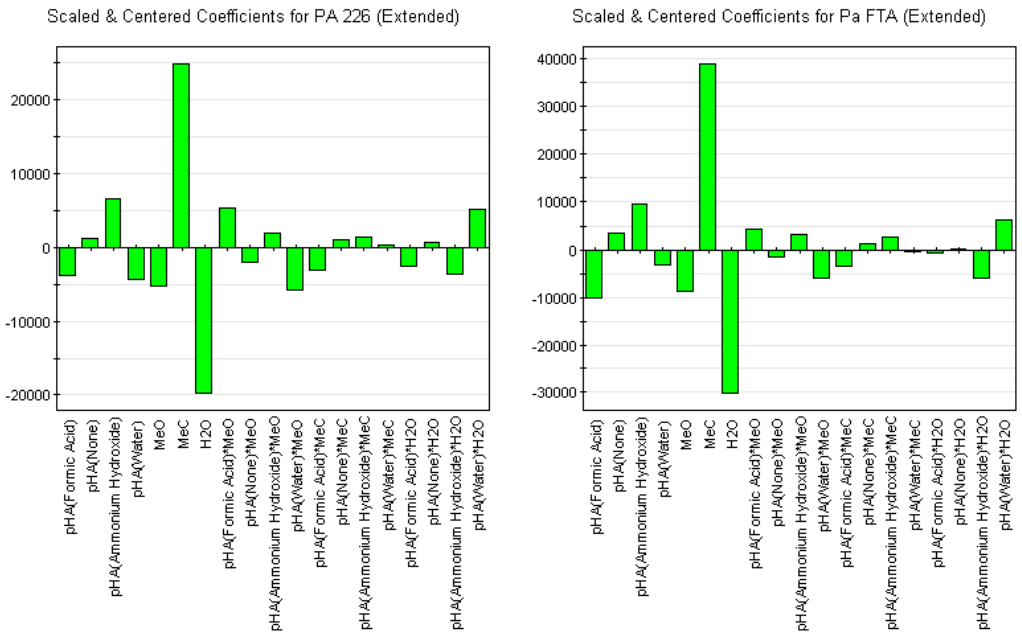


Figure 9-6; Coefficients plot of dried blood spot recovery screening test

9.4.1 The Effect of Extraction pH on Recovery of SARY

To further investigate the relationship between pH modification and the composition of acetonitrile in the overall extraction solvent, further experiments were designed using MODDE. A sample of SARY was prepared in human whole blood at 5000 ng/mL and spotted in 25 µL aliquots onto FTA® cards, allowed to dry and stored in a dessicator for at least 1 week. Analytical samples from 4.2 mm hole cuts were taken into polypropylene deep well microtitre plate (Porvair, Fareham, UK) using a BSD robot. Modification of pH in the first stage was done by adding 100 µL aliquots of 1M ammonium hydroxide (pH 11.5) 1 M formic acid (pH 2.1) and a neutral solution of 1M ammonium hydroxide adjusted to pH7. The microtitre plate was sealed and samples were mixed for 10 minutes on a MicroMix plate mixer. Following mixing, 100, 200 or 300 µL aliquots of acetonitrile were added and mixed on the plate mixer for a further 30 minutes. After extraction, an aliquot of the extracted sample was removed and added to a further microtitre plate and diluted with acetonitrile so that the final proportion of acetonitrile in each sample was equivalent. This was to equate total dilution of the extracted sample, to avoid solvent strength influencing injection volume or chromatographic extraction, and to prevent non-equivalent evaporation of sample volume on the autosampler prior to injection. The diluted extracted sample was analysed using the method described in chapter 8.

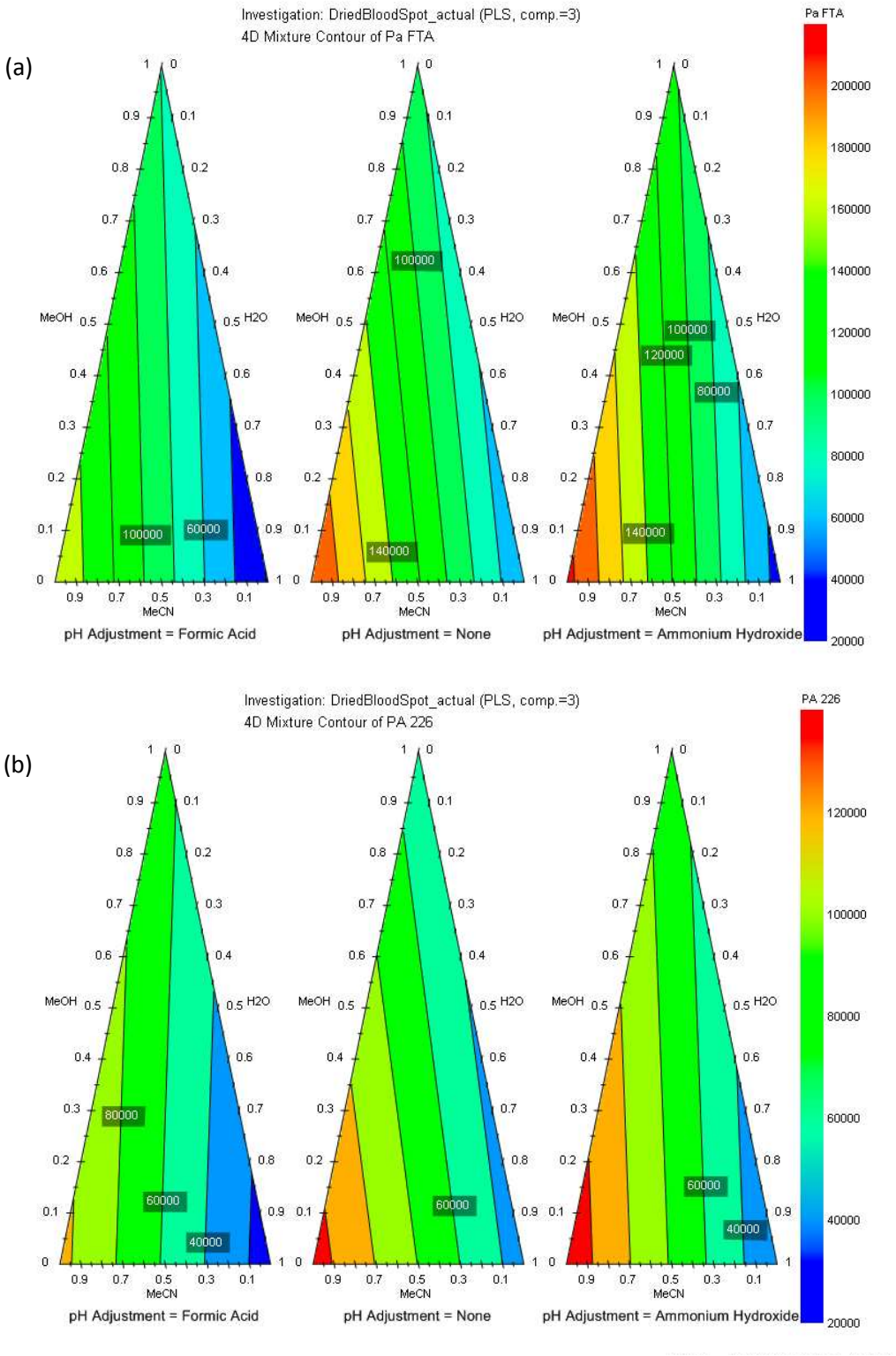


Figure 9-7; DBS extraction screening 4D contour plot for (a) FTA paper and (b) IDB226 paper

The contour map of the peak area recovery derived using MODDE software is represented in Figure 9-7 and shows the relationship derived from the data available. As suggested by previous screening experiments, extraction is favoured by basic pH modification although

the minima of the recovery surface plot is in the region around pH 5 and adjustment to lower pH also enhances recovery. Increasing the proportion of acetonitrile in the second stage of extraction relative to the aqueous solvent volume also increased recovery although the improvement is only slight, so not optimal, since large organic solvent proportions are unfavourable to avoid solvent composition modification required prior to chromatographic sample analysis.

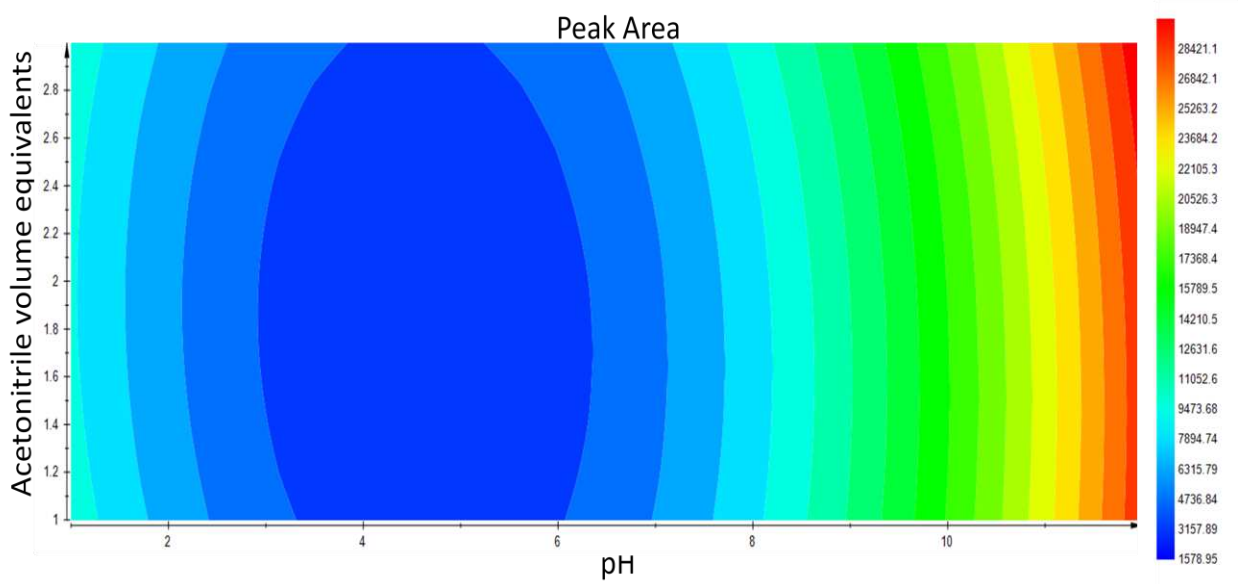


Figure 9-8; Contour plot of the relationship between pre-treatment buffer pH and acetonitrile elution solvent volume equivalents on SARY recovery from FTA paper

9.5 Lipid Recovery from Paper

9.5.1 Absolute Lipid Recovery

To investigate the effect of extraction conditions on the recovery of phospholipids, the MODDE analysis method relating pH modification and acetonitrile volume to recovery were repeated analysing human whole blood spotted onto FTA® paper as described in 9.4.1. The extracts were analysed by the phospholipid screening LC-MS method described in chapter 7. While all precursors of m/z = 184 Da were monitored, a chromatographic integration method was written to extract five of the most abundant lipid species as probe species.

Similar to the recovery relationship observed from SARY, neutral pH modification was unfavourable to recovery, while in most cases, increasing pH before extraction increased yield. Only in the case of lipid 4 (m/z = 667.5>184 Da) extraction recovery was greater under acidic conditions and lowest following increase in pH while in most cases, recovery

increased under very acidic conditions. These results conclude that firstly selective extraction of SARY from dried blood spots is unlikely to be achieved using the most optimum elution conditions for SARY, while the actual composition of phospholipid molecular species eluted from dried blood spots is variable depending on extraction conditions.

9.6 Lipid Distribution across the Paper

To evaluate the affinity of lipid recovery from paper and the potential impact of distribution effects observed visually in whole blood spotted onto certain paper matrices, a phospholipid test method was developed and applied to the analysis of probe phospholipids from blood spots. 100 µL of human whole blood was spotted onto various paper types and allowed to dry at room temperature before transfer to a desiccator for desiccated storage. Larger blood spots than those anticipated for practical use were employed to enable multiple cuts across the spot so that any distribution effects may be detected. The extraction method was as follows:

- 4.2 mm punch was taken using a BSD600 laser aligned robot punching tool and transferred to an eppendorf tube
- 50 µL acetonitrile added, sealed and mixed on shaker for 10 minutes
- 75 µL water added to prepare injection solvent, sealed and mixed on shaker for 10 minutes
- Tube stood for 5 minutes and extract removed to a microtitre plate for injection.
- 50 µL analysed by the selected lipids analytical method described in chapter 7.

The blood spots produced together with the position of sampling punch cuts across the spot are represented in Figure 9-10 together with the recovery of probe phospholipids represented in a chart below each blood spot. It can be seen that there is variability in the recovery of probe phospholipids from different paper types and as anticipated by the appearance of spotted blood, the distribution across the spots is greater in treated papers, particularly FTA elute® than untreated IDB226 paper. The practice of punching analytical samples directly from the centre of the spot facilitated by using laser alignment on the BSD600 robot is expected to standardise some distribution effects.

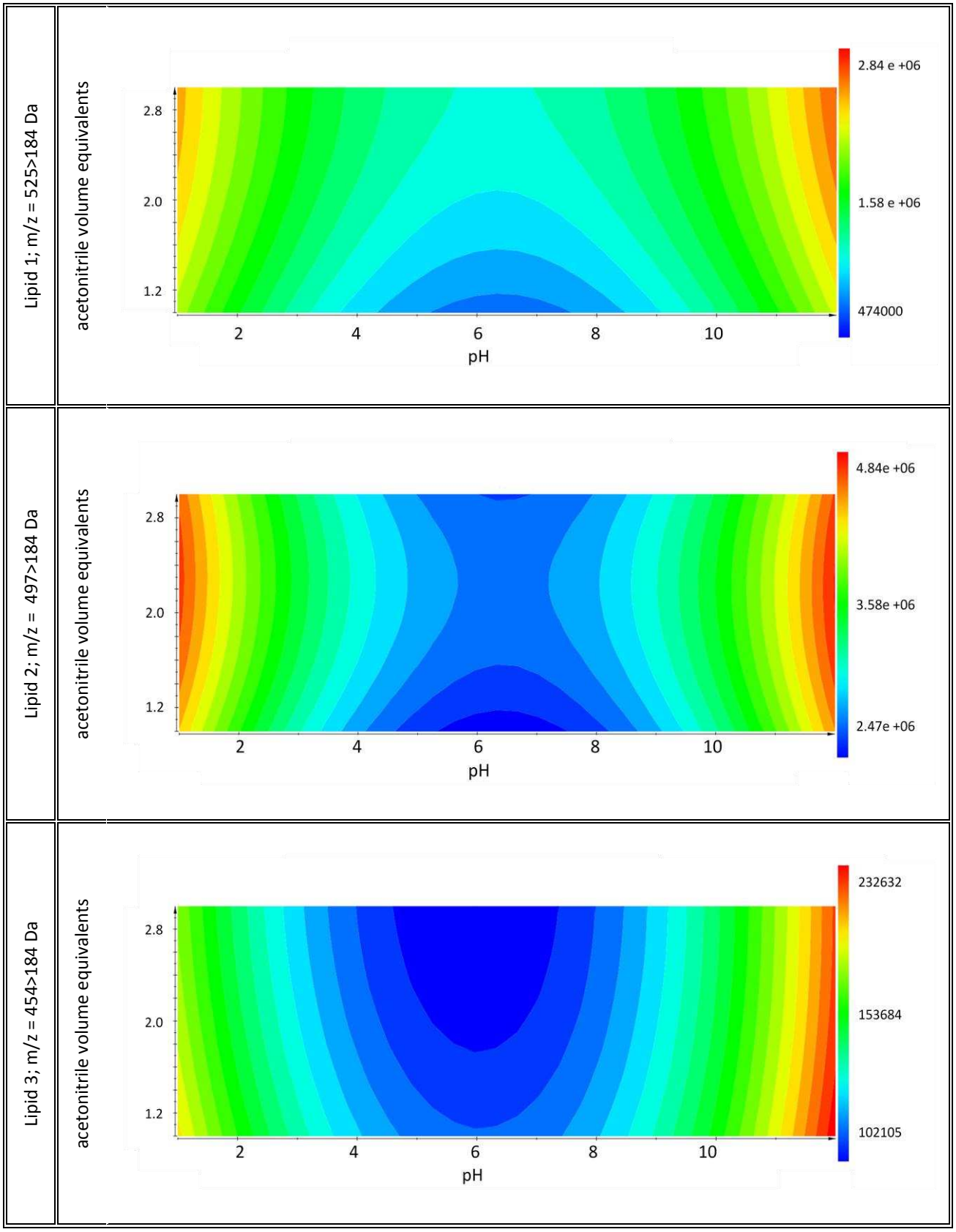


Figure 9-9; Contour plot of the relationship between pre-treatment buffer pH and acetonitrile elution solvent volume equivalents on the recovery from FTA paper of five different probe phospholipids.

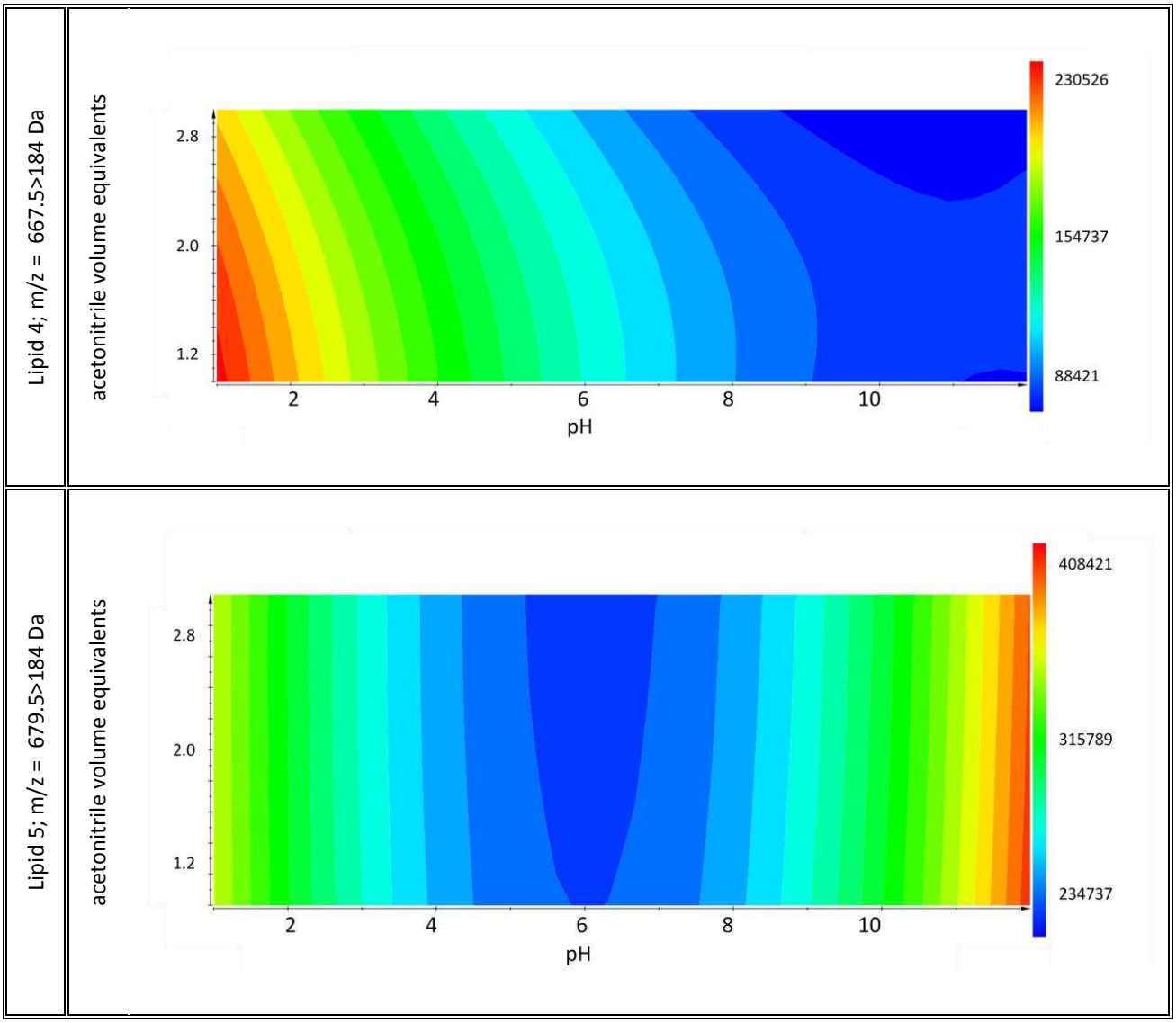


Figure 9-9 (continued); Contour plot of the relationship between pre-treatment buffer pH and acetonitrile elution solvent volume equivalents on the recovery from FTA paper of five different probe phospholipids.

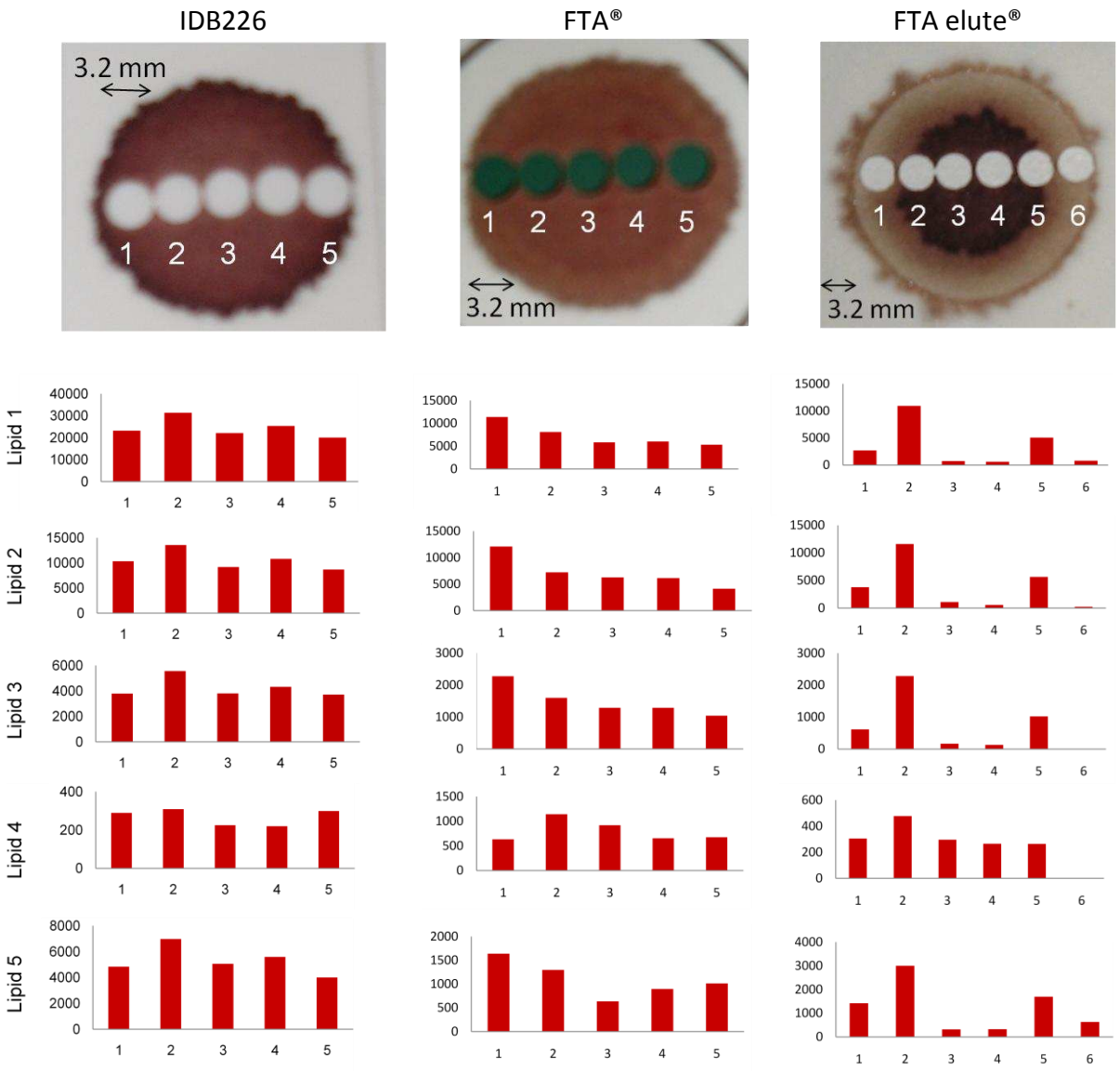


Figure 9-10; Probe phospholipids extracted from dried spots of human whole blood on 3 paper types; (left to right) i) IDB226, ii) FTA iii) FTA elute

9.7 Dried Blood Spot Discussion

A method for the analysis of SARY in whole blood was evaluated, factors influencing the recovery of the compound from dried blood spots on paper were investigated and the optimum extraction solvent appropriate to generate a sample for analysis by LC-MS was found to be acetonitrile. It was determined that previous pH adjustment to increase the pH of the sample enhanced the extraction using acetonitrile. Alternative paper media were investigated and the highest recovery when extracted under identical conditions was achieved from FTA® paper. FTA® paper is a chemically modified paper and while the chemistry of the treatment is proprietary, it is known to contain sodium dodecyl sulphate.

Since the compound is lipophilic, it is suggested that interaction between the compound and the alkyl chain of the dodecyl sulphate group associated with the paper and subsequent release into extraction solvent facilitates this. Alternative chemical modification of untreated papers prior to sampling has been shown to influence recovery and storage stability in lipophilic drugs. [158, 143] Since the log D of SARY is unchanged between pH 2 and pH 10, the variation of recoveries of the analyte from paper between these limits is attributed to interaction with the cellulose paper media (Figure 9-11) rather than the physicochemical properties of the analyte.

Analysis of glycerophospholipids revealed that recovery was additionally favoured by high or low pH, meaning that optimum conditions observed for recovery of the target analyte were also likely to recover phospholipids. It is also proposed that the affect of high or low pH on recovery is in part due to hydrolysis of the glycosidic linkages between saccharide monomers.

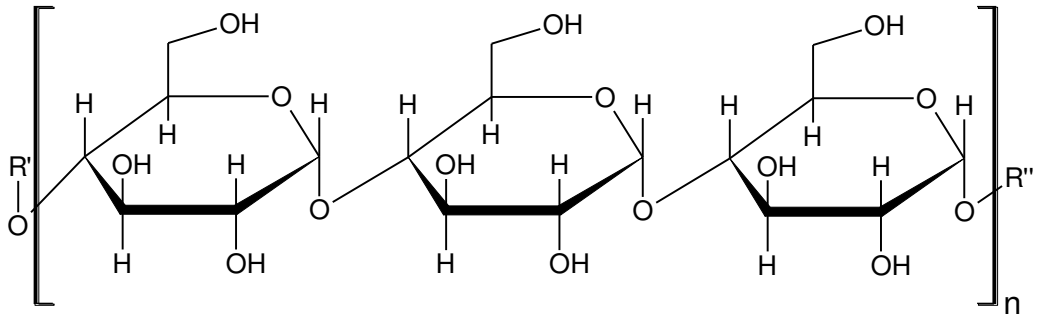


Figure 9-11; Monosaccharide composition of cellulose

The distribution of phospholipids across the blood spot formed by the addition of human whole blood treated and untreated papers was investigated and revealed a more uniform distribution across untreated paper than treated papers. This is attributed to rapid lysis of red blood cells on contact with the chemicals coating of the treated papers altering the homogeneity of the sample during application. It was shown that while the temperature of blood during application to the paper affected the speed formation of the absorbed spot due to changes in viscosity, there was no effect on the size of spot formed; therefore sample concentration density depending on blood handling immediately following sampling at body temperature.

10 Conclusions and Future Work

Conclusions

Initial evaluation of cross calibration was unsuccessful giving rise to a degree of bias in quantification which indicated that the assay was unsuitable for use with calibration samples prepared in a surrogate species. It was proposed that this effect was a result of inequivalent relative ionisation suppression of the drug molecule and its stable isotope labelled internal standard between plasma derived from different species. The phenomena was tested by means of a simple test of adding a solution containing a fixed ratio of drug and labelled internal standard to test samples of plasma matrix from different sources or species. The samples were prepared to contain the same amount of each component as present at the lower limit of quantification where the impact of ionisation suppression differences is predicted to be greatest. Following analysis of the prepared samples by the LC-MS/MS method, the calculated peak area ratios obtained for each test plasma source were compared to quantify the bias associated uniquely with matrix effect.

The presence of a region of ionisation suppression was identified co eluting with the analytes by application of a continuous post column infusion test to evaluate the effect of co-eluting compounds on the ionisation efficiency of the target analyte. Full scan mass spectra confirmed that the predominant species co eluting with the analyte were phosphotidylcholine species.

A Turboflow LC method was prepared to investigate the distribution of glycerophospholipids analysing precursors of m/z = 184 Da to identify the molecular masses of phosphotidylcholine and sphingomyelin membrane phospholipids which are abundant and diverse groups and which account for the greatest concentration of circulating plasma phospholipids. Comparison of the profiles obtained by analysing test samples from multiple species identified a species difference in phospholipid molecular species distribution. Furthermore, separation was observed between the distribution of phospholipids in samples taken from human volunteers from different ethnic groups using a partial least squared regression discriminative analysis investigation.

It is concluded that differences in phospholipid species distributions observed gave rise to matrix effect bias. One proposed mechanism arises from the formation of micelles as phospholipids elute from the analytical column containing a different composition of constituent molecules. This infers characteristic surface properties on the micelle which interact with the drug and internal standard discriminatively thereby forming an equilibrium which continues into the vapour phase within the source and a proportionate quantity of each analyte ion are inhibited from entering the gas phase for transferral to the mass analyser.

A second analytical method was investigated for a new compound with attention to resolution of the analyte and internal standard from endogenous phospholipids. On line extraction column chemistry and elution conditions and column wash were optimised to reduce the amount of lipids transferred onto the analytical column. The fixed ratio standards test at the lower limit of the assay was applied and the distribution of ratio values between test species were found to lie within 5% of the mean value.

Validation tests were applied to the method by using a human plasma derived calibration line to analyse samples prepared in rat and mouse plasma in three replicates of four concentration levels across the dynamic range of the assay on six separate occasions. The validation results were found to comply with regulatory requirements [98] that the mean calculated concentration at each level was within 15% of the nominal value and no more than 33% of individual values exceeded this limit. The method was then used to analyse samples taken following dosage to mice as part of a drug development study using human plasma calibration and compared with the results obtained from matrix matched calibration. It was demonstrated that the method using matrix matched or human surrogate plasma were equivalent for the quantification of SARY in incurred samples in mouse plasma. Furthermore, acceptability of incurred sample reproducibility was determined according to regulatory guidelines [227] using human plasma samples as a surrogate calibration source by comparison to analysis using calibration samples in mouse plasma.

A method was evaluated for the extraction and analysis of SARY in whole blood dried onto filter paper in the form of dried blood spots. The preliminary method involved cutting a

sample from the dried blood spot, extracting the analyte using acetonitrile and modifying solvent composition and analysis by the plasma Turboflow LC-MS/MS method. It demonstrated acceptable precision and bias and was shown to be robust for analysis of a maximum run size of 96 samples. Factors influencing the distribution of blood across different paper samples and recovery of both analyte molecule and probe phospholipids were investigated and discussed, differing distribution of phospholipids across each paper were identified by sampling punch position. Analysis of non-clinical samples in whole blood in the form of dried blood spots as an alternative to plasma as an exposure medium has been identified to make a significant contribution to the reduction and refinement of the use of animals in drug development studies. [180]

Future Work

To develop the project further, additional investigation into the elimination of phospholipids in the context of high throughput bioanalysis for pharmacokinetic studies would be recommended either in addition to or in place of solid phase extraction. One method would be liquid-liquid extraction with solid support in the form of diatomaceous earth materials or semi permeable membrane. A further method would be removal of phospholipids by aggregation involving the chelation of lanthanide salts.

The proposed mechanism of involvement of a micellar pseudo stationary phase in contribution to discriminative ionisation suppression of analyte by coeluting phospholipids would be investigated by the formation of micelles using a synthetic mixture of commercially available (lyso)phosphotidylcholine species which could be manipulated to coelute with a probe analyte (and isotopically labelled internal standard) and the micellar properties adjusted by alteration to the phospholipid composition.

The effect of storage conditions on the stability of analyte on cellulose filter paper and the influence of the stability of cellulose media and chemical paper treatments should be investigated in more long term studies. Recently, a new development in dried blood spot analysis involving glass fibre based filter papers for therapeutic monitoring analysis was discussed. Advantages to this method arise from the inert nature of glass fibre paper as opposed to contribution of cellulose hydrolysis at extremes of pH. pH could subsequently be more selectively adjusted to influence analyte recovery and furthermore, chemical

modification of the glass fibre paper could be investigated to influence binding or stability of analyte on glass fibre paper using aliphatic chain surfactants such as sodium dodecyl sulphate or proteinaceous material such as bovine serum albumin.

The use of fixed ratio standard solution approach in the early stages of method evaluation should be implemented with other analytes and used to assess the potential of bias caused by nonlinear ionisation efficiency in the presence of co eluting molecular species. This test may be used to investigate the effect on human diversity on the precision and accuracy of a clinical bioanalytical assay. Moreover it could be used to rapidly evaluate the potential to cross validate an assay for the analysis of animal samples using calibration samples from a surrogate source.

11 Bibliography

1. Adams, C. P.; Brantner, V. V. Estimating the cost of new drug development: Is it really $802 million? *Health Affairs* **2006,** *25* (2), 420-428.

2. Sanofi-Aventis. *Research and Development Brochure;* Paris, 2006.

3. Want, E.; Grieg, M.; Compton, B.; Bolanõs, B.; Siuzdak, G. Mass spectrometry in high throughput analysis. *Spectroscopy* **2003,** *17,* 663-680.

4. Gad, S. C., Ed. *Drug discovery handbook;* John Wiley & sons: Hoboken, 2005.

5. Nnane, I. P. Pharmacokinetics; Absorption, distribution, and elimination. In *Encyclopaedia of Analytical Science;* Elsevier, 2005.

6. Ng, R. *Drugs: From discovery to approval;* John Wiley & sons: Hoboken, 2004.

7. Tozer, T. N.; Rowland, M. *Introduction to Pharmacokinetics and Pharmacodynamics: The Quantitative Basis of Drug Therapy;* Lippincott Williams & Wilkins: Baltimore, 2006.

8. Berkow, R., Ed. *The Merck Manual;* Merck & Co: Pensylvania, 1997.

9. Lemberger, L. Pharmacokinetics and adverse reactions: An overview. In *Toxicokinetics and new drug development;* Yakobi, A., Ed.; Pergamon press: New York, 1989; pp 114-121.

10. Calcagno, A. M.; Siahaan, T. J. Physicological, biochemical and chemical barriers to oral drug delivery. In *Drug delivery: principals and applications;* Wang, B., Siahaan, T., Soltero, R., Eds.; John Wiley & sons: Hoboken, 2005; Chapter 2.

11. Brocks, D. R.; Mehvar, R. Rate and Extent of Drug Accumulation after Multiple Dosing Revisited. *Clinical Pharmacokinetics* **2010,** *49* (7), 421-438.

12. Akula, R.; Hasan, S.; Pipalla, R.; Ferguson, C. Noncompliance leading to drug accumulation resulting in phenytoin toxicity. *Journal of the National Medical Association* **2003,** *95* (12), 1201-1203.

13. Routeledge, P. A. The plasma protein binding of basic drugs. *British Journal of Clinical Pharmacology* **1986,** *22,* 499-506.

14. Lindup, W. E.; L'Eorme, M. C. Plasma protein binding of drugs. *British Medical Journal* **1981,** *282,* 212-214.

15. Jemal, M.; Xia, Y.-Q. LC-MS development strategies for quantitative bioanalysis. *Current Drug Metabolism* **2006,** *7,* 491-502.

16. Biddlecombe, R. A.; Pleasance, S. Automated protein precipitation by filtration in the 96-well format. *Journal of Chromatography B* **1999,** *734,* 257-265.

17. Polson, C.; Sarkar, P.; Incledon, B.; Raguvaran, V.; Grant, R. Optimisation of protein precipitation based on effectiveness of protein removal and ionisation effect in liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* **2003,** *785,* 263-275.

18. Souverain, S.; Rudaz, S.; Veuthey, J.-L. Matrix effect in LC-ESI-MS and LC-APCI-MS with off-line and on-line extraction procedures. *Journal of Chromatography A* **2004,** *1058* (1-2), 61-66.

19. James, C. Solid Phase Extraction. In *Principals and Practice of Bioanalysis;* Venn, R. F., Ed.; Taylor and Francis: London, 2000; Chapter 2.

20. Wells, D. A. Bioanalytical applications: Solid phase extraction. In *Encyclopedia of Separation Science;* Wilson, I. D., Ed.; Academic Press, 2000; pp 2142-2146.

21. Wells, D. A. Chapter 11 Solid-phase extraction: High throughput techniques. In *Progress in Pharmaceutical and Biomedical Analysis;* Elsevier: Amsterdam, 2003; Vol. 5, pp 361-432.

22. Haller, F.; Prehn, C.; Adamski, J. Quantification of steroids in human and mouse plasma using online solid phase extraction coupled to liquid chromatography tandem mass spectrometry, 2010. Nature Protocols. http://www.natureprotocols.com/2010/02/16/quantification_of_steroids_in.php (accessed September 12, 2010).

23. Kwok, W. H.; Leung, D. K. K.; Leung, G. N. W.; Wan, T. S. M.; Wong, C. H. F.; Wong, J. K. Y. Screening of drugs in equine plasma using automated on-line solid-phase extraction coupled with liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A* **2010,** *1217* (19), 3289-3296.

24. Rao, R. N.; Kumar, K. N.; Shinde, D. D. Determination of rat plasma levels of sertraline enantiomers using direct injection with achiral–chiral column switching by LC–ESI/MS/MS. *Journal of Pharmaceutical and Biomedical Analysis* **2010,** *52* (3), 398-405.

25. Wagner, M.; Bourgogne, E.; Varesio, E.; Hopfgartner, G. Quantitation of polar analytes using column-switching: Application to oxycodone and three metabolites in human plasma. *Journal of Chromatography B* **2010,** *878* (7), 637-644.

26. Bai, F.; Fraga, C. H.; Tagen, M.; Schaiquevich, P.; Hagedorn, N.; Stewart, C. F. Simultaneous determination of cyclophosphamide and carboxyethylphosphoramide mustard in human plasma using online extraction and electrospray tandem mass spectrometry (HTLC–ESI-MS/MS). *Journal of Chromatography B* **2009,** *877* (18), 1709-1715.

27. Zhou, Z. L.; Yu, X. Y.; Yang, M.; Mai, L. P.; Lin, Q. X.; Deng, C. Y.; Shan, Z. X.; Kuang, S. J.; Zhu, S. J.; Huang, X. Z. Improved liquid chromatography-tandem mass spectrometry method for the analysis of eptifibatide in human plasma. *Journal of Chromatography B* **2010,** *878* (23), 2094-2100.

28. Partani, P.; Gurule, S.; Khuroo, A.; Monif, T.; Bhardwaj, S. Liquid chromatography/electrospray tandem mass spectrometry method for the determination of cefuroxime in human plasma: Application to a pharmacokinetic study. *Journal of Chromatography B* **2010,** *878* (3), 428-434.

29. Partani, P.; Modhave, Y.; Gurule, S.; Khuroo, S.; Monif, T. Simultaneous determination of propranolol and 4-hydroxy propranolol in human plasma by solid phase extraction and liquid chromatography/electrospray tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **2009,** *50* (5), 966-976.

30. Serra, A.; Macià, A.; Romero, M.-P.; Salvado, M. J.; Bustos, M. J.; Fernández-Larrea, M.; Motilva, M.-J. Determination of procyanidins and their metabolites in plasma samples by improved liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (11), 1169-1176.

31. Deshpande, N. M.; Gangrade, M. G.; Kekare, M. B.; Vaidya, V. V. Determination of free and liposomal Amphotericin B in human plasma by liquid chromatography–mass spectroscopy with solid phase extraction and protein precipitation techniques. *Journal of Chromatography B* **2010,** *878* (3), 315-126.

32. Zhang, D.; Fu, Y.; Gale, J. P.; Aubry, A. F.; Arnold, M. A sensitive method for the determination of entecavir at picogram per milliliter level in human plasma by solid phase extraction and high-pH LC–MS/MS. *Journal of Pharmaceutical and Biomedical Analysis* **2009,** *49* (4), 1027-1033.

33. Lindegardh, N.; Tarning, J.; Toi, P. V.; Hien, T.; Farrar, J.; Singhasivanon, P.; White, N. J.; Ashton, M.; Day, N. P. J. Quantification of artemisinin in human plasma using liquid chromatography coupled to tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **2009,** *49* (3), 768-773.

34. Kannan, V.; Gadamsetty, D.; Rose, M.; Maria, S.; Mustafa, I.; Khedkar, A.; Dave, N.; Arumugam, M.; Iyer, H. Quantitative determination of oxytocin receptor antagonist atosiban in rat plasma by liquid chromatographytandem mass spectrometry. *Journal of Chromatography B* **2010,** *878* (15), 1069-1076.

35. Nagashima, T.; Aoyama, T.; Fukasawa, A.; Watabe, S.; Fukuda, N.; Ueno, T.; Sugiyama, H.; Nagase, H.; Matsumoto, Y. Determination of pyrrole-imidazole polyamide in rat plasma by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (11), 1070-1076.

36. Saracino, M. A.; Raggi, M. A. Analysis of soy isoflavone plasma levels using HPLC with coulometric detection in postmenopausal women. *Journal of Pharmaceutical and Biomedical Analysis* **2010,** *53* (3), 682-687.

37. Bu, W.; Sexton, H.; Fan, X.; Torres, P.; Houston, P. H.; Heyman, I.; Liu, L. The novel sensitive and high throughput determination of cefepime in mouse plasma by SCX-LC/MS/MS method following off-line µElution 96-well solid-phase extraction to support systemic antibiotic programs. *Journal of Chromatography B* **2010,** *878* (19), 1623-1628.

38. Suarez, M.; Romero, M. P.; Macia, A.; Valls, R. M.; Fernandez, S.; Sola, R.; Motilva, M. J. Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid-phase extraction plate and liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (32), 4097-4116.

39. Bhatt, M.; Shah, S.; Shivprakash. Development of a high-throughput method for the determination of ethosuximide in human plasma by liquid chromatography mass spectrometry. *Journal of Chromatography B* **2010,** *878* (19), 1605-1610.

40. Deng, P.; Chen, X.; Tang, Y.; Wang, Y.; Zhang, H.; Zhong, D. Determination of arotinoid acid in human plasma by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (27), 2983-2988.

41. Liu, K.; Dai, X.; Zhong, D.; Deng, P.; Ma, J.; Chen, X. Simultaneous determination of 6R-leucovorin, 6S-leucovorin and 5-methyltetrahydrofolate in human plasma using solid phase extraction and chiral liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (10), 902-910.

42. Britton, R. G.; Fong, I.; Saad, S.; Brown, K.; Steward, W. P.; Gescher, A.; Sale, S. Synthesis of the flavonoid 3',4',5'-trimethoxyflavonol and its determination in plasma and tissues of mice by HPLC with fluorescence detection. *Journal of Chromatography B* **2009,** *877* (10), 939-942.

43. Guilhaumou, R.; Solas, C.; Rome, A.; Giocanti, M.; Andre, N.; Lacarelle, B. Validation of an electrospray ionization LC/MS/MS method for quantitative analysis of vincristine in human plasma samples. *Journal of Chromatography B* **2010,** *878* (3), 423-427.

44. Valls, R. M.; Soler, A.; Girona, J.; Heras, M.; Romero, M. P.; Covas, M. I.; Sola, R.; Masana, L.; Motilva, M. J. Effect of the long-term regular intake of virgin olive oil on the phenolic metabolites in human fasting plasma. *Journal of Pharmaceutical and Biomedical Analysis* **2010,** *53* (1), 68-74.

45. McWhinney, B. C. The analysis of glucocorticoid steroids in plasma, urine and saliva by UPLC/MS/MS. *Clinica Chimica Acta* **2010,** *411* (11), 900-901.

46. Gordien, J. B.; Pigneux, A.; Vigouroux, S.; Tabrizi, R.; Accoceberry, I.; Bernadou, J. M.; Rouault, A.; Saux, M. C.; Breilh, D. Simultaneous determination of five systemic azoles in plasma by high-performance liquid chromatography with ultraviolet detection. *Journal of Pharmaceutical and Biomedical Analysis* **2009,** *50* (5), 932-938.

47. Gomes, N. A.; Laud, A.; Pudage, A.; Joshi, S. S.; Vaidya, V. V.; Tandel, J. A. Validated LC–MS/MS method for determination of Alverine and one of its hydroxy metabolites in human plasma along with its application to a bioequivalence study. *Journal of Chromatography B* **2009,** *877* (3), 197-206.

48. Levi, M.; Wuerzner, G.; Ezan, E.; Pruvost, A. Direct analysis of valsartan or candesartan in human plasma and urines by on-line solid phase extraction coupled to electrospray tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (10), 919-926.

49. Mercolini, L.; Gerra, G.; Consorti, M.; Somaini, L. R.; Raggi, M. A. Fast analysis of catecholamine metabolites MHPG and VMA in human plasma by HPLC with fluorescence detection and a novel SPE procedure. *Talanta* **2009,** *78* (1), 150-155.

50. Martens-Lobenhoffer, J.; Dautz, C.; Bode-Boger, S. M. Improved method for the determination of cyclic guanosine monophosphate (cGMP) in human plasma by LC–MS/MS. *Journal of Chromatography B* **2010,** *878* (3), 487-491.

51. Shakleya, D. M.; Huestis, M. A. Simultaneous and sensitive measurement of nicotine, cotinine, trans-3'-

hydroxycotinine and norcotinine in human plasma by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (29), 3537-3542.

52. Ding, X.; Chou, B.; Graham, R. A.; Cheeti, S.; Persey, S.; Matassa, L. C.; Reuschel, L. C.; Meng, S. A.; Liu, M.; Voelker, T. Determination of GDC-0449, a small-molecule inhibitor of the Hedgehog signaling pathway, in human plasma by solid phase extraction-liquid chromatographic-tandem mass spectrometry. *Journal of Chromatography B* **2010,** *878* (9), 785-790.

53. Kuhn, J.; Gotting, C.; Kleesiek, K. Simultaneous measurement of amiodarone and desethylamiodarone in human plasma and serum by stable isotope dilution liquid chromatography–tandem mass spectrometry assay. *Journal of Pharmaceutical and Biomedical Analysis* **2010,** *51* (1), 210-216.

54. Wu, Y. T.; Huang, C. M.; Lin, C. C.; Ho, W. A.; Lin, L. C.; Chiu, T. F.; Tarng, D. C.; Lin, C. H.; Tsai, T. H. Determination of melamine in rat plasma, liver, kidney, spleen, bladder and brain by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **2009,** *1216* (44), 7595-7601.

55. Chen, G.; Ren, L.; Yang, S. M.; Qiu, J.; Kamal-Eldin, A.; Lundstrom, K. Determination of androstenone levels in porcine plasma by LC-MS/MS. *Food Chemistry* **2010,** *122* (4), 1278-1282.

56. Liu, D.; Jiang, J.; Wang, P.; Feng, S.; Hu, P. Simultaneous quantitative determination of olmesartan and hydrochlorothiazide in human plasma and urine by liquid chromatography coupled to tandem mass spectrometry. *Journal of Chromatography B* **2010,** *878* (9), 743-748.

57. Locatelli, I.; Mrhar, A.; Grabnar, I. Simultaneous determination of risperidone and 9-hydroxyrisperidone enantiomers in human blood plasma by liquid chromatography with electrochemical detection. *Journal of Pharmaceutical and Biomedical Analysis* **2009,** *50* (5), 905-910.

58. Wei, X.; Yang, G.; Qi, L.; Chen, Y. Determination of nicardipine and amlodipine in human plasma using on-line solid-phase extraction with a monolithic weak cation-exchange column. *Talanta* **2009,** *77* (3), 1197-1202.

59. Bayliss, M. A. J.; Venn, R. F.; Edgington, A. M.; Webster, R.; Walker, D. K. Determination of a potent urokinase-type plasminogen activator, UK-356,202, in plasma at pg/mL levels using column-switching HPLC and fluorescence detection. *Journal of Chromatography B* **2009,** *877* (3), 121-126.

60. de Jong, W. H. A.; Smit, R.; Bakker, S. J.; de Vries, E. G. E.; Kema, I. P. Plasma tryptophan, kynurenine and 3-hydroxykynurenine measurement using automated on-line solid-phase extraction HPLC–tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (7), 603-609.

61. Mitrowska, K.; Posyniak, A.; Zmudzki, J. Multiresidue method for the determination of nitroimidazoles and their hydroxy-metabolites in poultry muscle, plasma and egg by isotope dilution liquid chromatography–mass spectrometry. *Talanta* **2010,** *81* (4), 1273-1280.

62. Kirsch, S. H.; Knapp, J. P.; Geisel, J.; Herrmann, W.; Obeid, R. Simultaneous quantification of S-adenosyl methionine and S-adenosyl homocysteine in human plasma by stable-isotope dilution ultra performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (30), 3865-3870.

63. Blackwell, S.; St. J. O'Reilly, D.; Talwar, D. K. HPLC analysis of asymmetric dimethylarginine (ADMA) and related arginine metabolites in human plasma using a novel non-endogenous internal standard. *Clinica Chimica Acta* **2009,** *401* (1), 14-19.

64. Mercolini, L.; Mandrioli, R.; Iannello, C.; Matrisciano, F.; Nicoletti, F.; Raggi, M. A. Simultaneous analysis of diazepam and its metabolites in rat plasma and brain tissue by HPLC-UV and SPE. *Talanta* **2009,** *80* (1), 279-285.

65. Chytil, L.; Matouakova, O.; Cerná, O.; Pokorna, P.; Vobruva, V.; Perlik, F.; Slana, Y. O. Enantiomeric determination of tramadol and O-desmethyltramadol in human plasma by fast liquid chromatographic technique coupled with mass spectrometric detection. *Journal of Chromatography B* **2010,** *878* (3), 481-486.

66. Kingback, M.; Josefsson, M.; Karlsson, L.; Ahlner, J.; Bengtsson, F.; Kugelberg, F. C.; Carlsson, B. Stereoselective determination of venlafaxine and its three demethylated metabolites in human plasma and whole blood by liquid chromatography with electrospray tandem mass spectrometric detection and solid phase extraction. *Journal of Pharmaceutical and Biomedical Analysis* **2010,** *53* (3), 583-590.

67. Xie, R.; Wen, J.; Wei, H.; Fan, G.; Zhang, D. High-throughput determination of faropenem in human plasma and urine by on-line solid-phase extraction coupled to high-performance liquid chromatography with UV detection and its application to the pharmacokinetic study. *Journal of Pharmaceutical and Biomedical Analysis* **2010,** *52* (1), 114-121.

68. Wang, Q.-Q.; Xianga, S.-S.; Jia, Y.-B.; Ou, L.; Chen, F.; Song, H. F.; Liang, Q.; Ju, D. An improved on-line solid phase extraction coupled HPLC–MS/MS system for quantification of Sifuvirtide in human plasma. *Journal of Chromatography B* **2010,** *878* (21), 1893-1898.

69. Saber, A. L. On-line solid phase extraction coupled to capilary LC-ESI-MS for determination of fluoxetine in human blood plasma. *Talanta* **2009,** *78* (1), 295-299.

70. Xu, Y.; Willson, K. J.; Anderson, M. D. G.; Musson, D. G.; Miller-Stein, C. M.; Woolfe, E. J. Elimination of diastereomer interference to determine Telcagepant (MK-0974) in human plasma using on-line turbulent-flow technology and off-line solid-phase extraction coupled with liquid chromatography/tandem mass spectrometry. *Journal of Chromatography B* **2009,** *877* (16), 1634-1642.

71. Gillings, N. A restricted access material for rapid analysis of [11C]-labeled radiopharmaceuticals and their metabolites in plasma. *Nuclear Medicine and Biology* **2009,** *36* (8), 961-965.

72. Borges, N. C.; Astigarraga, R. B.; Sverdloff, C. E.; Galvina, P. R.; da Silva, W. M.; Rezende, V. M.; Moreno, R. A. A novel and sensitive method for ethinylestradiol quantification in human plasma by high-performance liquid chromatography coupled to atmospheric pressure photoionization (APPI) tandem mass spectrometry: Application to a comparative pharmacokinetics study. *Journal of Chromatography B* **2009,** *877* (29), 3601-3609.

73. Nguyen, A. Q. N.; Theoret, Y.; Chen, C.; Denault, A.; Varin, F. High performance liquid chromatography using UV detection for the quantification of milrinone in plasma: Improved sensitivity for inhalation. *Journal of*

Chromatography B **2009,** *877* (7), 657-660.

74. Wu, X.; Liu, L.; Zhang, M.; Wu, D.; Wang, Y.; Sun, Y.; Fawcett, J. P.; Gu, J.; Zhang, J. Simultaneous analysis of isomers of escin saponins in human plasma by liquid chromatography–tandem mass spectrometry: Application to a pharmacokinetic study after oral administration. *Journal of Chromatography B* **2010,** *878* (11), 861-867.

75. Wang, Y.; Du, R. Simultaneous extraction of trace organophosphorous pesticides from plasma sample by automated solid phase extraction and determination by gas chromatography coupled with pulsed flame photometric detector. *Forensic Science International* **2010,** *198* (1), 70-73.

76. Hasler, F.; Kuznetsova, O. F.; Krasikova, R. N.; Cservenyak, T.; Quednow, B. B.; Vollenweider, F. X.; Ametamey, S. M.; Westera, G. GMP-compliant radiosynthesis of [18F]altanserin and human plasma metabolite studies. *Applied Radiation and Isotopes* **2009,** *67* (4), 598-601.

77. Saracino, M. A.; Petio, C.; Vitali, M.; Franchini, L.; Raggi, M. A. Determination of orphenadrine plasma levels using HPLC with diode array detection and a novel solid-phase extraction procedure in psychiatric patients. *Journal of Pharmaceutical and Biomedical Analysis* **2009,** *50* (3), 501-506.

78. Davies, A.; Hayes, A. K.; Knight, K.; Watmough, S. J.; Pirmohamed, P.; Clark, R. E. Simultaneous determination of nilotinib, imatinib and its main metabolite (CGP-74588) in human plasma by ultra-violet high performance liquid chromatography. *Leukaemia Research* **2010,** *34* (6), 702-707.

79. Boos, K.-S. A short course in LC-MS adequate cleanup of biofluids. *21st Montreux LC-MS Symposium*, Montreux, 2004.

80. Chen, L.; Wang, H.; Zeng, Q.; Xu, Y.; Sun, L.; Xu, H.; Ding, L. On-line Coupling of Solid-Phase Extraction to Liquid Chromatography—A Review. *Journal of Chromatographic Science* **2009,** *47* (10), 614-623.

81. Poole, C. F. New trends in solid-phase extraction. *Trends in Analytical Chemistry* **2003,** *22* (6), 362-373.

82. Pinkerton, T. C.; Hagestam, I. H. Internal surface reversed-phase silica supports for liquid chromatography. *Analytical Chemistry* **1985,** *57* (8), 1757-1763.

83. Souverain, S.; Rudaz, S.; Veuthey, J.-L. Restricted access materials and large particle supports for on-line sample preparation: an attractive approach for biological fluids. *Journal of Chromatography B* **2004,** *801,* 141-156.

84. Desilets, C. P.; Rounds, M. A.; Regnier, F. E. Semipermeable-surface reversed-phase media for high-performance liquid chromatography. *Journal of Chromatography A* **1991,** *544,* 25-39.

85. Boos, K.-S.; Grimm, C.-H. High-performance liquid chromatography integrated solid-phase extraction in bioanalysis using restricted access precolumn packings. *Trends in Analytical Chemistry* **1999,** *18* (3), 195-180.

86. Puhlmann, A.; Dülffer, T.; Kobold, U. Multidimensional high-performance liquid chromatography on Pinkerton ISRP and RP18 columns: direct serum injection to quantify creatinine. *Journal of Chromatography:*

Biomedical Applications **1992,** *581* (1), 129-133.

87. Hermansson, J.; Grahn, A.; Hermansson, I. Direct injection of large volumes of plasma/serum on a new biocompatible extraction column for the determination of atenolol, propranolol and ibuprofen: Mechanisms for the improvement of chromatographic performance. *Journal of Chromatography A* **1998,** *797* (1-2), 251-263.

88. Rbeida, O.; Christiaens, B.; Hubert, P.; Lubda, D.; Boos, K.-S.; Chromen, J.; Chiap, P. Evaluation of a novel anion-exchange restricted-access sorbent for on-line sample clean-up prior to the determination of acidic compounds in plasma by liquid chromatography. *Journal of Chromatography A* **2004,** *1030* (1-2), 95-102.

89. Ayrton, J.; Dear, G. J.; Leavens, W. J.; Mallet, D. N.; Plumb, R. S. The use of turbulent flow chromatography/mass spectrometry for the rapid, direct analysis of a novel pharmaceutical compound in plasma. *Rapid Communications in Mass Spectrometry* **1998,** *18* (11), 1953-1958.

90. Edge, T. Are turbulent times ahead for bionalysis? *Laboratory Automation* **2002,** *27,* 70.

91. Edge, T. Turbulent flow chromatography in bioanalysis. In *Handbook of Analytical Separations;* Wilson, I. D., Ed.; Elsevier Science: Amsterdam, 2003; Vol. 4; Bioanalytical Separations, pp 91-128.

92. Quinn, H. M.; Takarewski, J. J. High Performance Liquid Chromatography Method and Apparatus. Patent No. WO97/16724, May 09, 1997.

93. Ettre, L. S. Comissions on chromatography and other analytical separations and analytical nomenclature. *Pure and Applied Chemistry* **1993,** *65* (4), 819-872.

94. Ardrey, B. *Liquid-Chromatography-Mass Spectrometry: An Introduction;* John Wiley & sons: Chichester, 2003.

95. Dean, J. R. *Practical Inductively Coupled Plasma Spectroscopy;* John Wiley & sons: Hoboken, 2005.

96. Matuszewski, B. K. Standard line slopes as a measure of a relative matrix effect in quantitative HPLC–MS bioanalysis. *Journal of Chromatography B* **2006,** *830* (2), 185-390.

97. Bakes, D. Strategy for the development of quantitative analytical procedures. In *Principals and Practice of Bioanalysis;* Venn, R. F., Ed.; Taylor and Francis: London, 2000; Chapter 16.

98. Food and Drug Administration. *Guidance for Industry - Bioanalytical Method Validation;* United States Department of Health and Human Services: Rockville, 2001.

99. King, R.; Bonfiglio, R.; Fernandez-Metzler, C.; Miller-Stein, C.; Olah, T. Mechanistic investigation of ionisation suppression in electrospray ionisation. *Journal of the American Association of Mass Spectrometry* **2000,** *11,* 942-950.

100. Jessome, J. J.; Volmer, D. A. Ion suppression; a major concern in mass spectrometry. *LCGC* **2006,** *24,* 498-510.

101. van Eekhaut, A.; Lanckmans, K.; Sarre, S.; Smolders, I.; Michotte, Y. Validation of bioanalytical LC-MS/MS assays; Evaluation of matrix effects. *Journal of Chromatography B* **2009,** *877,* 2198-2207.

102. Bruins, C. H. P.; Jeronimus-Stratingh, C. M.; Ensing, K.; van Dongen, W. D.; de Long, G. J. On line coupling of solid phase extraction with mass spectrometry for the analysis of biological samples I. Determination of clenbutarol in urine. *Journal of Chromatography A* **1999,** *863,* 115-122.

103. Mallet, C. R.; Lu, Z.; Mazzeo, J. R. A study of ion suppression effects in electrospray ionisation from mobile phase additives and solid-phase extracts. *Rapid Communications in Mass Spectrometry* **2004,** *18,* 49-58.

104. Matuzewsk, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. Matrix effect in quantitative LC/MS/MS analysis of biological fluids; a method for the determination of finasteride in human plasma at picogram per millilitre concentrations. *Analytical Chemistry* **1998,** *70,* 882-889.

105. Enke, C. J.; Jackson, G. S.; Constantantopoulis, T. L. Effects of salt concentration on analyte response using electrospray ionisation mass spectrometry. *Journal of the American Society of Mass Spectrometry* **1999,** *10,* 625-634.

106. Liesener, A.; Karst, U. Turbulent flow chromatography for the reduction of matrix effects in electrospray ionization mass spectrometry-based enzyme assays. *Journal of Separation Science* **2005,** *28* (14), 1658 - 1665.

107. King, R. C.; Bonfoglio, R.; Olah, T. V.; Merkle, K. The effects of sample preparation methods on the variability of electrospray ionisation response for model drug compounds. *Rapid Communications in Mass Spectrometry* **1999,** *13,* 1175-1185.

108. Williams, E. R.; Iavarone, A. T.; Udekwu, O. A. Buffer Loading for Counteracting Metal Salt-Induced Signal Suppression in Electrospray Ionisation. *Analytical Chemistry* **2004,** *76,* 3944-3950.

109. Mei, H.; Nardo, C.; Xu, X.; Hsei, Y.; Wang, S.; Ng, K.; Korfmacher, W. A. Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery. *Rapid Communications in Mass Spectrometry* **2003,** *17,* 97-103.

110. Beneijts, T.; Dams, R.; Lambert, W.; De Leenher, A. Countering matrix effects in environmental liquid chromatography–electrospray ionization tandem mass spectrometry water analysis for endocrine disrupting chemicals. *Journal of chromatography A* **2004,** *1029,* 153-159.

111. Weaver, R.; Rilet, R. J. Identification of ion suppression effects on pharmacokinetic parameters by polyethylene glycol 400. *Rapid Communications in Mass Spectrometry* **2006,** *20,* 2559-2564.

112. Larger, P. J.; Breda, M.; Fraier, D.; Hughes, H.; James, C. A. Ion-suppression effects in liquid chromatography-tandem mass spectrometry due to a formulation agent, a case study in discovery bioanalysis. *Journal of Pharmaceutical and Biomedical Analysis* **2005,** *39,* 206-216.

113. Korfmacher, W. A.; Xu, X.; Wang, S.; Zhou, Q.; Wang, G.; Broske, L.; Pena, A. A study of common discovery dosing formulation components and their potental for causing time-dependant matrix effects in high-

performance liquid chromatography tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **2005,** *19,* 2643-2650.

114. Leverence, R.; Avery, M. J.; Kavetskaia, O.; Bi, H.; Hop, C.; Arkady, G. Signal Suppression/enhancement in HPLC-ESI-MS/MS from concomitant medications. *Biomedical Chromatography* **2007,** *21,* 1143-1150.

115. Karnes, H. T.; Ismaiel, O. A.; Halquist, M. S.; Elmalmy, M. Y.; Shalby, A. Monitoring phospholipids for assessment of matrix effects in liquid chromatography-tandem mass spectrometry method for hydrocodone and pseudoephidrine in human plasma. *Journal of Chromatography B* **2007,** *259,* 84-93.

116. Little, J. L.; Wempe, M. F.; Buchanan, C. M. Liquid chromatography-mass spectrometry/mass spectrometry method for drug metabolism studies: Examining lipid matrix ionisation effects in plasma. *Journal of Chromatography B* **2006,** *833,* 219-230.

117. Sojo, L. E.; Lum, G.; Chee, P. Internal standard signal suppression by co-eluting analyte in isotope dilution LC-ESI-MS. *The Analyst* **2003,** *128,* 51-54.

118. Liang, H. R.; Foltz, R. L.; Meng, M.; Bennet, P. Ionisation enhancement in atmospheric pressure chemical ionisation and suppression in electrospray ionisation between target drugs and stable-isotope-labelled internal standards in quantitative liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **2003,** *17,* 2815-2821.

119. Voet, D.; Voet, J. G. Lipids and Membranes. In *Biochemistry;* Wiley: New York, 1990; pp 271-313.

120. Gunstone, F. D. *Fatty acid and lipid chemistry;* Blackie academic and professional: Glasgow, 1996.

121. Wolf, C.; Quinn, P. J. Lipidomics; Practical aspects and applications. *Progress in Lipid Research* **2008,** *47,* 15-36.

122. Millne, S.; Ivanova, P.; Forrester, J.; Brown, H. A. Lipidomics: An analysis of cellular lipids by ESI-MS. *Methods* **2006,** *39,* 92-103.

123. Domingues, M. R. M.; Reis, A.; Pedro, D. Mass Spectrometry analysis of oxidised phospholipids. *Chemistry and Physics of Lpids* **2008,** *156* (1-2), 1-12.

124. Han, X. Lipidomics: Developments and applications. *Journal of chromatography B* **2009,** *877,* 2663.

125. Hu, C.; van der Heijden, R.; Wang, M.; van der Greef, J.; Hankemeier, T.; Xu, G. Analytical strategies in lipidomics and applications in disease biomarker discovery. *Journal of Chromatography B* **2009,** *877,* 2836-2846.

126. Jia, L.; Wang, C.; Zhao, Z.; Lu, X.; Xu, G. Metabolomic identification of potential phospholipid biomarkers for chronic glomeluritis by using high performance liquid chromatography - mass spectrometry. *Journal of Chromatography B* **2007,** *860,* 134-140.

127. Taylor, L. A.; Arends, A. K.; Unger, C.; Massing, U. Plasma lyso-phosphatidylcholine is decreased in cancer patients with weight loss and activated inflamatory status. *Lipids in health and disease* **2007,** *6* (17).

128. Kuliszkiewicz-Janus, M.; Tuz, M. A. T.; Baczynski, S. Application of 31PNMR to the analysis of phospholipid changes in plasma of patients with acute leukaemia. *Biochemica et Physica Acta* **2005,** *1737,* 11-15.

129. Tanford, C. *The hydrophobic effect: formation of micelles and biological membranes,* 2nd ed.; Wiley Interscience Publications: Michigan, 1980; p 109.

130. Guthrie, R. Blood screening for phenylketonuria. *Journal of the American Medical Association* **1961,** *178,* 863.

131. Guthrie, R.; Suzi, A. A simple Phenylalanine method for detecting Phenylketonuria in large populations of newborn infants. *Paediatrics* **1963,** *32,* 338-343.

132. United Kingdon Newborn Screening Programme Centre. http://newbornbloodspot.screening.nhs.uk/aboutus. http://newbornbloodspot.screening.nhs.uk/aboutus (accessed September 12, 2010).

133. Rashed, M. S.; Rahbeeni, Z.; Ozand, P. T. Application of electrospray tandem mass spectrometry to neonatal screening. *Seminars in Perinatology* **1999,** *23* (2), 183-193.

134. Zoppa, M.; Gallo, L.; Zacchello, F.; Giordano, G. Method for the quantification of underivatised amino acids on dry blood spots from newborn screening by HPLC-ESI-MS/MS. *Journal of Chromatography B* **2006,** *831,* 267-273.

135. Li, P. K.; Lee, J. T.; Conboy, K. A.; Ellis, E. F. Fluorescence polarisation immunoassay for theophylline modified for use with dried blood spots on filter paper. *Clinical Chemistry* **1986,** *32* (3), 552-555.

136. Tawa, R.; Matsunaga, H.; Fujimoto, T. High-performance liquid chromatographic analysis of aminoglycoside antibiotics. *Journal of Chromatography A* **1998,** *812,* 141-150.

137. Koal, T.; Burhenne, H.; Römlng, R.; Svoboda, M.; Resch, K.; Kaever, V. Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **2005,** *19,* 2995-3001.

138. AbuRuz, S.; Millership, J.; McElnay, J. Dried blood spot liquid chromatography assay for therapeutic drug monitoring of metformin. *Journal of Chromatography B* **2006,** *832,* 202-207.

139. Hoogstanders, K.; van der Heijden, J.; Christiaans, M.; Edelbroek, P.; van Hooff, J. P.; Stolk, L. M. L. Therapeutic monitoring of tacrolimus with the dried blood spot method. *Journal of Pharmaceutical and Biomedical Analysis* **2007,** *44,* 658-664.

140. Lejeune, D.; Souletie, I.; Houzé, S.; Le bricon, T.; Le bras, J.; Gourmel, B.; Houzé, P. Simultaneous determination of monodesethylchloroquine, chloroquine, cycloguanil and proguanil on dried blood spots by reverse-phase liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis* **2007,** *43,* 1106-

1115.

141. Janson, A.; Gustafsson, L. L.; Mirghani, R. A. High-performance liquid chromatographic method for the determination of quinine and 3-hydroxyquinine in blood samples dried on filter paper. *Journal of Chromatography B* **2003,** *795,* 151-156.

142. Malm, M.; Lindegårdh, N.; Bergqvist, Y. Automated solid-phase extraction method for the determination of piperaquine in capillary blood applied onto sampling paper by liquid chromatography. *Journal of Chromatography B* **2004,** *809,* 43-49.

143. Blessborn, D.; Römsing, S.; Annerberg, A.; Sundquist, D.; Björkman, A.; Lindegarth, N.; Bergqvist, Y. Development and validation of an automated solid-phase extraction and liquid chromatographic method for determination of lumefantrne in capillary blood on sampling paper. *Journal of Pharmaceutical and Biomedical Analysis* **2007,** *45,* 282-287.

144. Beaudette, P.; Bateman, K. P. Discovery stage pharmacokinetics using dried blood spots. *Journal of Chromatography B* **2004,** *809,* 153-158.

145. NC3Rs. General Principals; Volume of Blood to be Removed. http://www.nc3rs.org.uk/bloodsamplingmicrosite/page.asp (accessed September 7th, 2010).

146. Spooner, N.; Barfield, M.; Lad, R.; Parry, S.; Fowles, S. Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies. *Journal of Chromatography B* **2008,** *870,* 32-37.

147. Spooner, N.; Ladd, R.; Barfield, M. Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method. **2009,** *81* (4), 1557-1563.

148. Liang, X.; Li, Y.; Barfield, M. Study of dried blood spots technique for the determination of dextromethorphan and its metabolite dextrorphan in human whole blood by LC–MS/MS. *Journal of Chromatography B* **2009,** *877* (8-9), 799-806.

149. Spooner, N.; Ramakrishnan, Y.; Barfield, M.; Dewit, O.; Miller, S. Use of DBS sample collection to determine circulating drug concentrations in clinical trials: practicalities and considerations. *Bioanalysis* **2010,** *2* (8), 1515-1522.

150. Smith, L. M.; Burgoyne, L. A. Collecting, archiving and processing DNA from wildlife samples. *BMC Ecology* **2004,** *4* (4).

151. Hsiao, K.; Lin, H.; Pan, H.; Lai, T.; Chen, S.; Jou, S.; Chiu, Y.; Wu, M.; Lin, C.; Li, S. Application of FTA sample collection and DNA purification system on the determination of CTG trinucleotide repeat size by PCR-based Southern blotting. **1999,** *13* (4), 188-193.

152. Ndunguru, J.; Taylor, N. J.; Yadav, J.; Legg, J. P.; Aly, H.; Aveling, T.; Thompson, G.; Fauquet, C. M. Application

of FTA technology for sampling, recovery andmolecular characterization of viral pathogens and virus-derived transgenes from plant tissues. *Virology Journal* **2005,** *2* (45).

153. Duscher, G.; Paschke, R.; Wille-Piazzai, W.; Anja, J. Parasites on paper - The use of FTA elute (R) for the detection of Dirofilaria repens microilariae in canine blood. *Veterinary parasitology* **2009,** *161,* 349-351.

154. de Vargas Wolfgramm, E.; Magri de Carvalho, F.; da Costa Aguiar, V. R.; De Nadai Sartori, M. P.; Hirschfeld-Campolongo, G. C. R.; Tsustsumida, W. M.; Drumond Louro, I. Simplified buccal DNA extraction with FTA (R) elute cards. *Forensic science international: Genetics* **2009,** *3,* 125-127.

155. Allanson, A. L.; Cotton, M. M.; Tettley, J. N. A.; Boyter, A. C. Determination of rifampicin in human plasma and blood spots by high performance liquid chromatography with UV detection: a potential method for therapeutic drug monitoring. *Journal of Pharmaceutical and Biomedical Analysis* **2007,** *44,* 963-969.

156. Peng, S.-H.; Segura, J.; Farré, M.; De La Torre, X. Oral testosterone administration detected by testosterone glucuronidation measured in blood spots dried on filter paper. *Clinical Chemistry* **2000,** *46* (4), 515-522.

157. Schulze, A.; Schmidt, C.; Kohnmüller, D.; Hoffmann, G. F.; Mayatepek, E. Accurate measurement of free carnitine in dried blood pots by isotope-dilution electrospray tandem mass spectrometry without butylation. *Clinica Chimica Acta* **2003,** *335,* 137-145.

158. van der Heijden, J.; de Beer, Y.; Hoogstanders, K.; Christiaans, M.; de Jong, G. J.; Neef, C.; Stolk, L. Therapeutic drug monitoring of everolimus using the dried blood spot method in combination with liquid chromatography-mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **2009,** *50,* 664-670.

159. Green, M. D.; Mount, D. L.; Nettey, H. High-performance liquid chromatographic assay for the simmultaneous determination of sulfadoxine and pyrimethamine from whole blood dried onto filter paper. *Journal of Chromatography B* **2002,** *767,* 159-162.

160. Damen, C. W. N.; Rosing, H.; Schellens, J. H. M.; Beijen, J. H. Application of dried blood spots combined with high-performance liquid chromatography coupled with electrospray ionisation tandem mass spectrometry for simultaneous quantification of vincristine and actinomycin-D. *Analytical and Bioanalytical Chemistry* **2009,** *394,* 1171-1182.

161. Liu, G.; Patrone, L.; Snapp, H. M.; Batog, A.; Valentine, J.; Cosma, G.; Tymiac, A.; Ji, Q. C.; Arnold, M. E. Evaluating and defining sample preparation procedures for DBS LC-MS/MS assays. *Bioanalysis* **2010,** *2* (8), 1405-1414.

162. Rosunblum, W. I. Invitro measurements of the effects of anticoagulants on the flow properties of blood: The relationship of these effects to red cell shrinkage. **1968,** *31* (2), 234-241.

163. Mayer, G. E. Blood viscosity in healthy subjects and patients with coronary heart disease. *Canadian Medical Association Journal* **1964,** *91,* 951-954.

164. Reinhart; Reinhart, W. H.; Haeberli, A.; Stark, J.; Straub, P. W. Influence of blood withdrawl and anticoagulant

type on clotting activity, hematologic data, and certain rheologic measurements. *Journal of Laboratory Clinical Medicine* **1990,** *115* (1), 98-103.

165. Wells, R. E.; Merrill, E. W. Influence of flow properties of blood upon viscosity - hematocrit relationships. *Journal of Clinical Investigation* **1962,** *41* (8), 1291-1598.

166. Mei, J. V.; Alexander, J. R.; Adam, B. W.; Hannon, W. H. Use of filter paper for the collection and analysis of human whole blood specimens. *The Journal of Nutrition* **2001,** *131,* 1631-1636.

167. Holub, M.; Tuschl, K.; Ratschmann, R.; Strnadová, K. A.; Mühl, A.; Heinze, G.; Sperl, W.; Bodamer, O. A. Influence of hematocrit and localisation of punch in dried blood spots on levels of amino acids and acylcarnitines measured by tandem mass spectrometry. *Clinica Chimica Acta* **2006,** *373,* 27-31.

168. Ren, X.; Paehler, T.; Zimmer, M.; Guo, Z.; Zane, P.; Emmons, G. Impact of various factors on radioactivity distribution in different DBS papers. *Bioanalysis* **2010,** *2* (8), 1469-1475.

169. Flemming, D. O., Hunt, D. L., Eds. *Biological Safety: Principals and Practices,* 3rd ed.; ASM Press: Washington, 2000.

170. Furr, K., Ed. *CRC Handbook of Laboratory Safety,* 5th ed.; CRC Press: Boca Raton, Fl, 2000.

171. WHO. *Manual on the management, maintenance and use of blood cold chain equipment;* World Health Organisation: Geneva, 2005.

172. Evengard, B.; von Sydow, M.; Ehrnst, A.; Pehrson, P. O.; Lundbherg, P.; Linder, E. Filter paper sampling of blood infected with HIV: effect of heat on antibody activity and viral infectivity. *British Medical Journal* **1988,** *297,* 1178.

173. Bond, W. W.; Favero, M. S.; Petersen, N. J.; Gravelle, C. R.; Ebert, J. W.; Maynard, J. E. Survival of hepatitis B virus after drying and storage for one week. *Lancet* **1981,** *317* (8219), 550-551.

174. Parker, S. P.; Cubitt, W. D. The use of dried blood spot sample in epidemiological studies. *Journal of Clinical Pharmacology* **1999,** *52,* 633-639.

175. Resnick, L.; Veren, K.; Salahuddin, S. Z.; Tondreu, S.; Markham, P. D. Stability and inactivation of HTLV-III/LAV under clinical and laboratory environments. *Journal of the American Medical Association* **1986,** *255* (14), 1887-1891.

176. International Air Transport Association. Infectious Substances Guidance Document, 2010. IATA. http://www.iata.org/SiteCollectionDocuments/Documents/Guidance_Doc62DGR_51.pdf (accessed September 12, 2010).

177. de Castro Toledo, A. C.; Januário, J. N.; Silva Rezende, R. M.; Siqueira, A. L.; de Mello, B. F.; Fialho, É. L.; Ribero, R. A.; da Silva, H. L.; Pires, É. C.; Simóes, T. C.; Greco, D. B. Dried blood spots as a practical and inexpensive source for human immunodeficiency virus and hepatitis C virus surveillance. *Memórias Do*

Instituto Oswaldo Cruz **2005,** *100* (4), 365-370.

178. Ivers, L. C.; Smith Fawzi, M. C.; Mann, J.; Jerome, J.-G.; Raymonville, M.; Mukherjee, J. S. Overseas processing of dried blood spot samples for timely diagnosis of HIV in Haitian infants. *Revista Panamerica de Salud Pública* **2008,** *24* (5), 331-335.

179. Brambilla, D.; Jennings, C.; Aldrovandi, G.; Bremer, J.; Comeau, A. M.; Cassol, S. A.; Dickover, R.; Jackson, J. B.; Pitt, J.; Sullivan, J. L.; Butcher, A.; Grosso, L.; Reichelderfer, P.; Fiscus, S. Multicenter evaluation of use of dried blood and plasma spot specimens in quantitative assays for Human Immunodeficiency Virus RNA: Measurement, Precision and RNA stability. *Journal of Clinical Microbiology* **2003,** *41* (5), 1888-1893.

180. Lovatt, C. A.; Dopson, W. J.; Barfield, M.; Spooner, N.; Sparrow, S. J.; Fowless, S. Opportunities for REDUCTION and REFINEMENT: Use of Dried Blood Spots for Generation of Toxicokinetic Data, 2010. NC3Rs. http://www.nc3rs.org.uk/downloaddoc.asp?id=874&page=973&skin=0 (accessed July 07, 2010).

181. Anderson, B. J.; Potts, A. L.; Herd, D. W. Problems and pitfalls performing pharmacokinetic studies in children. *Paediatric and Perinatal Drug Therapy* **2007,** *8* (1), 4-17.

182. *Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals;* ICH harmonised tripartite guideline; International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH): Geneva, Switzerland, 2009.

183. World Medical Association. Declaration of Helsinki (1964). *British Medical Journal* **1996,** *313* (7070), 1448-1449.

184. Secretary of State for the Home Department. *Animals (Scientific Procedures) Act;* UK Act of Parliament; The Staionary Office: London, 1986.

185. Council of Europe. European convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS No. 123) Guideline for the accomodation and care of animals, 2006. Council of Europe. http://conventions.coe.int/Treaty/en/Treaties/html/123.htm (accessed September 17, 2010).

186. Shah, V. P.; Midha, K. K.; Dighe, S.; McGilveray, I. J.; Skelly, J. P.; Yacobi, A.; Layloff, T.; Vishwanathan, C.; Cook, T.; McDowall, R. D.; Pittman, K. A.; Spector, S. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. *Journal of Pharmaceutical Science* **1992,** *81* (3), 309-312.

187. National Centre for Reduction Refinement and Replacement of Animals in Research. Annual Report, 2009. NC3Rs. http://www.nc3rs.org.uk/downloaddoc.asp?id=1105&page=201&skin=0 (accessed September 12, 2010).

188. Sanofi-Aventis. Charter for the humane care and use of laboratory animals, 2007. research and development. http://en.sanofi-aventis.com/binaries/animal_charter_en_tcm28-21508.pdf (accessed August 17, 2010).

189. Flaherty, J. M.; Connolly, P. D.; Decker, E. R.; Kennedy, S. M.; Ellefson, M. E.; Reagen, W. K.; Szostek, B. Quantitative determination of perfluoric acid in serum and plasma by liquid chromatography tandem mass

spectrometry. *Journal of Chromatography B* **2005,** *819,* 329-338.

190. Jemal, M.; Schuster, A.; Whigan, D. B. Liquid chromatography/tandem mass spectrometry methods for quantification of mevalonic acid in human plasma and urine: method validation, demonstration of using a surrogate analyte and demonstration of unacceptable matrix effect in spite of use of stable. *Rapid Communications in Mass Spectrometry* **2003,** *17* (15), 329-338.

191. Beharry, M. DBS: a UK (MHRA) regulatory perspective. *Bioanalysis* **2010,** *2* (8), 1363-1364.

192. Serradeil-Le Gal, C.; Valette, G.; Foulon, L.; Germain, G.; Advenier, C.; Nline, E.; Bardou, M.; Martinole, J.-P.; Pouzet, B.; Raufaste, D.; Garcia, C.; Double-Cazanave, E.; Pauly, M.; Pascal, M.; Barbier, A.; Scatton, B.; Maffraud, J.-P.; Le Fur, G. SSR126768A (4-Chloro-3-[(3R)-5-chloro-1-(2,4-dimethyoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl]-N-ethyl-N-(3-pyridinylmethyl)benzamide hydrochloride): A new selective and orally active oxytocin receptor antagonist for the prevention of pre-term l. *Journal of Pharmacology and Experimental Therapeutics* **2003,** *319* (1), 414-424.

193. Fura, A.; Harper, T. W.; Zhang, H.; Fung, L.; Shyu, W. C. Shift in pH of biological fluids during storage and processing: effect on bioanalysis. *Journal of Pharmaceutical and Biomedical Analysis* **2003,** *32* (3), 513-522.

194. Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS.. *Analytical Chemistry* **2003,** *75* (13), 3019-3030.

195. The Organisation for Economic Co-operation and Development. *OECD Principals on Good Laboratory Practice and compliance monitoring ENV/MC/CHEM(98)17;* The Organisation for Economic Co-operation and Development: Paris, 1998.

196. Xu, X.; Mei, H.; Wang, S.; Zhou, Q.; Wng, G.; Broske, L.; Pena, A.; Korfmacher, W. A. A Study of common discovery dosing formulation components and their potential for causing time-dependant matrix effects in high-performance liquid chromatography tandem mass spectrometry assays. *Rapid Communications in Masss Spectrometry* **2005,** *19* (18), 2643-2650.

197. Wang, S.; Cryonak, M.; Yang, E. Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. *Journal of Pharmaceutical and Biomedical Analysis* **2007,** *43* (2), 701-707.

198. Avery, M. J. Quantitative characterization of differential ion suppression on liquid chromatography/atmospheric pressure ionization mass spectrometry bioanalytical methods. *Rapid Communications in Mass Spectrometry* **2003,** *17,* 197-201.

199. Jemal, M.; Schuster, A.; Whigan, D. B. Liquid chromatography/tandem mass spectrometry methods for quantitation of mevalinc acid in human plasma and urine: method validation, demovstration of using a surrogate analyte, and demonstration of unacceptable matrix effect in spite of stable isotope. *Rapid Communications in Mass Spectrometry* **2003,** *17,* 1723-1734.

200. Liang, H. R.; Foltz, R. L.; Meng, M.; Bennet, P. Ionization enhancement in atmospheric pressure chemical

ionization and suppression in electrospray ionization between target drugs and stable-isotope-labeled internal standards in quantitative liquid chromatography tandem mass spectrometry assays. *Rapid Communications in Mass Spectrometry* **2003,** *17* (24), 2815-2282.

201. Zhang, G.; Wujcik, C. E. Overcoming ionisation effects through chromatography: A case study for the ESI-LC-MS/MS quantitation of a hydrophobic therapeutic agent in human serum using a stable-label internal standard. *Journal of Chromatography B* **2009,** *877,* 2003-2010.

202. Stovkis, E.; Rosling, H.; Beijnen, J. Stable isotopically labeled internal standards in quantitative bioanalysis using liquid chromatography/mass spectrometry: necessity or not? *Rapid Communications in Mass Spectrometry* **2005,** *19* (3), 401-407.

203. Siuzdak, G.; Bothner, B. Gas-Phase Micelles. *Angewandte Chemie International Edition in English* **1995,** *34* (18), 2053-2055.

204. James, P. F.; Perugini, M. A.; O'Hair, R. A. J. Sources of artefacts in the electrospray ionisation mass spectra of saturated diacylglycerophosphocholines: From condensed phase hydrolysis reactions through to gas phase intercluster reactions. *Journal of the American Society of Mass Spectrometry* **2006,** *17,* 384-394.

205. Beckman Coulter. Bioresearch Literature. Beckman Coulter. http://www.beckmancoulter.com/literature/Bioresearch/360643-CEPrimer1.pdf (accessed September 12, 2010).

206. Muijsellaar, P. G.; Otsuka, K.; Terabe, S. Micelles as pseudo stationary phases in micellar electrokinetic chromatography. *Journal of Chromatography A* **1997,** *780,* 41-61.

207. Dobashia, A.; Onoa, T.; Haraa, S.; Yamacuchi, J. Enantioselective hydrophobic entanglement of enantiomeric solutes with chiral functionalized micelles by electrokinetic chromatography. *Journal of Chromatography A* **1990,** *480,* 413-420.

208. Kitagawa, F.; Hasegawa, T.; Taji, H.; Onoue, S.; Tsuda, Y.; Otsuka, K. Fundamental Studies on Electrokinetic Chromatography with PEGylated Phospholipid Micelles. *Analytical Sciences* **2008,** *24,* 155-159.

209. Holland, L. A.; Leigh, A. M. Bilayered phospholipid micelles and capillary electrophoresis: A new additive for electrokinetic chromatography. *Electrophoresis* **2003,** *24* (17), 2935 - 2939.

210. Skeaff, C. M.; Hodson, L.; McKenzie, J. E. Dietary induced changes in fatty acid composition of human plasma, platelet, and erythrocyte lipids follow a similar time course. *The Journal of Nutrition* **2005,** *136,* 565-569.

211. Hwang, D. H.; Boudreau, M.; Chanmugam, P. Dietary (n=3) fatty acids alter fatty acid composition and prostaglandin synthesis in rat testis. *The Journal of Nutrition* **1988,** *118,* 1173-1178.

212. Hwang, D. H.; Boudreau, M.; Chanmugam, P. Dietary linoleic acid and longer chain n-3 fatty acids: comparison of effects on arachidonic acid metabolism in rats. *The Journal of Nutrition* **1988,** *118,* 427-437.

213. Ohvo-Rekilä, H.; Ramstedt, B.; Leppimäki, P. Phospholipid interactions with cholesterol in membranes. *Progress in lipid research* **2002,** *41,* 66-97.

214. Nelson, G. J. The phospholipid composition of plasma in various mammalian species. *Lipids* **1967,** *2* (4), 323-328.

215. Umetrics. SIMCA-P User Guide. www.umetrics.com. http://www.umetrics.com/Content/Document%20Library/Files/UserGuides-Tutorials/SIMCA-P_12_UG.pdf (accessed September 17, 2010).

216. Bloor, W. R. Lipemia. *The Journal of Biological Chemistry* **1921,** *41* (1), 201-227.

217. Gordon, R. S.; Cherkes, A. Unesterified fatty acid in human blood plasma. *Journal of Clinical Investigation* **1956,** *35,* 206-212.

218. Tan, C. E.; Tai, E. S.; Tan, C. S.; Chia, K. S.; Lee, J.; Chew, S. K.; Ordovas, J. M. APOE polymorphism and lipid profile in three ethnic groups in the Singapore polulation. *Atherosclerosis* **2003,** *170* (2), 253-260.

219. van Eijsen, M.; Hornstra, G.; van der Wal, M. F.; Bonsel, G. J. Ethnic differences in early pregnancy maternal n-3 and n-6 fatty acid concentrations: an explorative analysis. *British Journal of Nutrition* **2009,** *101,* 1761-1768.

220. Metcalf, P. A.; Sharret, A. R.; Folsom, A. R.; Duncan, B. B.; Patsch, W.; Hutchinson, R. G.; Szklo, M.; Davis, C. E.; Tyroler, H. A. African American-White differences in lipids, lipoproteins, and apolipoproteins, by educational attainment, among middle-aged adults: the atherosclerosis risk in communities study. *American Journal of Epidemiology* **1998,** *148* (8), 750-760.

221. Young, T. K.; Gerrard, J. M.; O'Neil, J. D. Plasma phospholipid fatty acids in the central Canadian Arctic: biocultural explanations for ethnic differences. *American Journal of Physical Anthropology* **1999,** *109* (1), 9-18.

222. International Conference on Harmonisation. Note for guidance on ethnic factors on the acceptability of foreign clinical data CPMP/ICH/289/95, 1998. European Medical Association. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002842.pdf (accessed September 12, 2010).

223. Food and Drug Administration. *Guidance for Industry - Collection of race and ethnicity data in clinical trials;* United States Department of Health and Human Services: Rockville, 2005.

224. Sanofi-Aventis, International Patent.

225. Bansal, S.; DeStefano, A. Key elements of bioanalytical method validation for small molecules. *Journal of the American Association of Pharmaceutical Science* **2007,** *9* (1), 109-114.

226. Miller, J. M.; Miller, J. C. Significance tests. In *Statistics for Analytical Chemistry,* 2nd ed.; Ellis Horwood, 1992;

pp 53-77.

227. Rossi, M. L.; Haughey, D.; Jardieu, P.; Devanarayan, V. Confirmatory Reanalysis of Incurred Bioanalytical Samples. *Journal of the American Association of Pharmaceutical Science* **2007,** *9* (3), 336-343.

228. Timmerman, P.; Luedtke, S.; van Amsterdam, P.; Brudny-Kloeppel, M.; Lausecker, B. Incurred sample reproducability: views and recommendations from the European Bioanalytical Forum. *Bioanalysis* **2009,** *1* (6), 1049-1056.

APPENDICES

	Issue	Article	Organic Mobile Phase	Salt		Acid/Base		
1	26	3	Acetonitrile	Ammonium acetate	10 mM	-	-	-
2		4	Methanol	Formic acid	0.1 %	-	-	-
3		8	Acetonitrile/ Methanol	Formic acid	0.05 %	-	-	-
4		9	Acetonitrile	Ammonium acetate	10 mM	-	-	-
5		12	Acetonitrile	Ammonium acetate	5 mM	Formic acid	0.2 %	-
6	25	2	Acetonitrile	Ammonium acetate	10 mM	-	-	-
7		5	Acetonitrile	-	-	Formic acid	0.01 %	
8		9	Acetonitrile	Ammonium formate	5 mM	-	-	
9		10	Methanol	Ammonium formate	3.5 mM	Formic acid	-	3.5
10		12	Acetonitrile	-	-	Formic acid	0.1 %	-
11	24	2	Acetonitrile	-	-	Formic acid	0.1 %	-
12		7	Methanol	Ammonium acetate	20 mM	-	-	-
13		13	Methanol	Ammonium acetate	10 mM	Acetic acid	0.1 %	-
14		15	Acetonitrile	-	-	Formic acid	0.01 %	-
15		16	Acetonitrile	Ammonium formate	1 mM	Formic acid	-	3
16		17	Acetonitrile	-	-	Formic acid	0.05 %	-
17		18	Acetonitrile	-	-	Formic acid	0.05 %	-
18	23	5	Acetonitrile	-	-	-	-	-
19		6	Acetonitrile	Ammonium formate	20 mM	formic acid	0.1 %	-
20		9	Acetonitrile	Ammonium acetate	10 mM	formic acid	0.1 %	-
21		10	Methanol	-	-	formic acid	0.1 %	-
22		11	Acetonitrile	-	-	formic acid	0.1 %	-
23		16	Acetonitrile	-	-	formic acid	0.5 %	-
24		19	Methanol	-	-	formic acid	1 %	-
25	22	5	Methanol	-	-	formic acid	0.05 %	-
26		8	Acetonitrile	-	-	formic acid	0.5 %	-
27		9	Methanol	Ammonium acetate	2 mM	formic acid	0.15 %	-
28		13	Methanol	Ammonium bicarbonate	10 mM	-	-	9
29		17	Acetonitrile	-	-	formic acid	0.1 %	-

	Issue	Article	Organic Mobile Phase	Salt		Acid/Base		
30	21	18	Acetonitrile	-	-	formic acid	0.1 %	-
31		19	Acetonitrile	-	-	formic acid	0.1 %	-
32		24	Acetonitrile	-	-	-	-	-
33		26	Methanol	-	-	formic acid	0.05 %	-
34		27	Methanol	-	-	formic acid	0.1 %	-
35	20	13	Acetonitrile	Ammonium formate	1 mM	Formic acid	-	3
36	19	5	Acetonitrile	-	-	formic acid	0.05 %	-
37		10	Methanol	Ammonium acetate	2 mM	-	-	-
38		11	Acetonitrile	Ammonium acetate	10 mM	-	-	7.1
39		26	Acetonitrile	Ammonium acetate	10 mM	-	-	-
40		27	Acetonitrile	-		-	-	-
41		30[A]	Acetonitrile	Ammonium acetate	10 mM	-	-	-
42		33	Acetonitrile	-	-	Formic acid	0.1 %	-
43	15-16	7	Acetonitrile/ Methanol	Ammonium acetate	20 mM			-
44		8	Acetonitrile	-	-	Acetic acid Trifluoroacetic acid	1 % 0.05 %	-
45		14	Acetonitrile	-	-	Formic acid	0.2 %	-
46		20	Acetonitrile/ Methanol	ammonium formate	10 mM	Formic acid	0.2 %	-
47		22	Methanol	Ammonium acetate	10 mM	Formic acid	2 %	-
48		25	Acetonitrile	-	-	-	-	-
49		26	Methanol	-	-	Acetic acid	2 %	
50	13-14	6	Acetonitrile/ Methanol	-	-	-	-	-
51		9	Acetonitrile	Ammonium acetate	10 mM	Acetic acid	-	3.7
52		11	Acetonitrile	Ammonium acetate	2 mM	-	-	-
53		13	Acetonitrile	Ammonium acetate	10 mM	-	-	-
54		15	Acetonitrile/ Methanol	Ammonium acetate	10 mM	Formic acid	0.1 %	-
55	11-12	4	Acetonitrile/	Ammonium	10 mM	-	-	pH

	Issue	Article	Organic Mobile Phase	Salt		Acid/Base		
			Methanol	acetate				6.8
56		7	Acetonitrile/ Methanol	Ammonium acetate	40 mM	-	-	-
57		9	Acetonitrile	-	-	Formic acid	0.1 %	-
58	9-10	2	Methanol	Ammonium acetate	10 mM	Formic acid	0.001 %	4.7
59		4	Acetonitrile/ Methanol	-	-	Formic acid	0.05 %	-
60		10	Acetonitrile	-	-	Formic acid	0.1 %	-
61		13	Acetonitrile	-	-	Formic acid	0.5 %	-
62		15	Methanol	-	-	-	-	-
63		16	Methanol	Ammonium formate	10 mM	-	-	-
64	7-8	2	Acetonitrile	Ammonium acetate	10 mM	-	-	-
65		7	Acetonitrile	-	-	Formic acid	0.1 %	-
66		9	Methanol	Ammonium formate	10 mM	Formic acid	-	3.5
67		10	Methanol	-	-	Acetic acid	0.3 %	-
68		11	Methanol	Ammonium acetate	10 mM	-	-	-
69		12	Methanol	Ammonium acetate	10 mM	Formic acid	0.1 %	-
70	5-6	3	Acetonitrile	Ammonium acetate	10 mM	-	-	5
71		4	Methanol	Ammonium acetate	10 mM	-	-	-
72		8	Methanol	Ammonium acetate	1 mM	-	-	-
73		13	Acetonitrile	-	-	Formic acid	0.25 %	-
74	3-4	8	Methanol	-	-	Formic acid	0.45 %	-
75		11	Acetonitrile	-	-	Formic acid	0.1 %	-
76		14	Methanol	Ammonium formate	10 mM	-	-	3
77		16	Methanol/ Acetonitrile	-	-	acetic acid	0.1 %	-
78		19	Acetonitrile	-	-	Formic acid	0.1 %	-
79		21	Acetonitrile	-	-	Acetic acid	1 %	4
80		22	Acetonitrile	Ammonium acetate	0.2 mM	-	-	-
81		23	Acetonitrile	-		Formic acid	0.1 %	-
82		32	Methanol	Ammonium bicarbonate	15 mM	-	-	10.5

	Issue	Article	Organic Mobile Phase	Salt		Acid/Base		
83		34	Methanol	Ammonium acetate	5 mM	Formic acid	0.1 %	-
84		35	Acetonitrile	-	-	Formic acid	0.5 %	-
85	1	4	Acetonitrile	-	-	Formic acid	0.1 %	-
86		5	Acetonitrile	-	-	Formic acid	0.1 %	-
87		6	Methanol	-	-	Formic acid	0.1 %	-
88		8[B]	Acetonitrile	Ammonium acetate	1 %	Acetic acid	0.035 %	-
89		13[C]	Acetonitrile	Ammonium acetate	10 %	Formic acid		6.5
90		18	Acetonitrile	-	-	Formic acid	0.2 %	-

A : Including 10% methanol

B : Including 0.002% trifluoracetic anhydride

C : Including 4% isopropyl alcohol

Appendix 1; Journal of Chromatography B 866 Volumes 1 to 26: review of mobile phase composition in published analytical methods for analysis small molecule pharmaceuticals in plasma by LC-MS

Sample	Calculated Concentration (ng/mL)		% Recovery (human /mouse)	%Difference
	Mouse Plasma Calibration	Human Plasma Calibration		
1	24200	27700	114	14.5
2	25800	29700	115	15.1
3	8530	7490	87.8	-12.2
4	1980	1790	90.4	-9.60
5	1470	1410	95.9	-4.08
6	1610	1490	92.5	-7.45
7	795	634	79.7	-20.3
8	1090	953	87.4	-12.6
9	953	774	81.2	-18.8
10	1090	950	87.2	-12.8
11	593	443	74.7	-25.3
12	907	860	94.8	-5.18
13	690	610	88.4	-11.6
14	791	641	81.0	-19.0
15	752	549	73.0	-27.0
16	121	103	85.1	-14.9
17	118	101	85.6	-14.4
18	80.9	71.4	88.3	-11.7
19	11200	12700	113	13.4
20	11300	11200	99.1	-0.88
21	12000	11500	95.8	-4.17
22	1330	961	72.3	-27.7
23	2800	2640	94.3	-5.71
24	2090	1770	84.7	-15.3
25	1410	1260	89.4	-10.6
26	1330	1290	97.0	-3.01
27	1410	1370	97.2	-2.84
28	901	730	81.0	-19.0
29	835	768	92.0	-8.02
30	703	592	84.2	-15.8
31	765	700	91.5	-8.50
32	906	843	93.0	-6.95
33	828	727	87.8	-12.2
34	46.5	44.9	96.6	-3.44
35	58.0	51.5	88.8	-11.2
36	80.0	74	92.5	-7.50
37	29.7	25.2	84.8	-15.2
38	37.0	28.8	77.8	-22.2
	Average		89.9	
	Standard Deviation		9.91	
	% Coefficient of Variation		11.0	
	% Bias		-10.1	

Appendix 2; Reproducibility of analysis of SARY in mouse plasma samples from dosed animals analysed using calibration samples prepared in mouse or human plasma.